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Biology of *Pseudomonas stutzeri*

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INTRODUCTION

Pseudomonas stutzeri was first described by Burri and Stutzer in 1895 (55). van Niel and Allen, in 1952 (371), precisely defined its phenotypic features and discussed its definitive designation as *Pseudomonas stutzeri* by Lehmann and Neumann (196). In spite of marked differences from the type strain of the genus, the sequence similarities of the rRNAs, demonstrated initially by DNA-rRNA hybridization, show the legitimacy of the inclusion of *P. stutzeri* in the genus *Pseudomonas*. Strains of the species have been identified among denitrifiers found in natural materials. Their inclusion in the phenotypic studies carried out by Stanier et al. in 1966 (340) demonstrated that, in addition to their typical colonies, the strains are nutritionally versatile, using some carbon compounds seldom utilized by other pseudomonads (e.g., starch, maltose, and ethylene glycol). Variations in DNA sequences, as shown by the results of DNA-DNA hybridization experiments, were demonstrated in the early studies of Palleroni et al., in 1970 (251). Work performed in recent years has clearly established firm bases for grouping the strains into a number of genomic variants (genomovars) that are phylogenetically closely related. Some strains have received particular attention because of specific metabolic properties (such as denitrification, degradation of aromatic compounds, and nitrogen fixation). Furthermore, some strains have been shown to be naturally transformable and have been studied extensively for their capacities for transformation. *P. stutzeri* is distributed widely in the environment, occupying diverse ecological niches, and has also been isolated as an opportunistic pathogen from humans. Based on results obtained in recent years, the biology of this species is discussed.

DEFINITION OF THE SPECIES AND DIFFERENTIATION FROM OTHER *PSEUDOMONAS* SPECIES

Definition

Pseudomonas stutzeri is a member of the genus *Pseudomonas* sensu stricto. It is in group I of Palleroni's DNA-rRNA homology group within the phylum *Proteobacteria* (252, 253). *P. stutzeri* is now recognized as belonging to the class *Gammaproteobacteria*. Phylogenetic studies of *P. stutzeri* strains' 16S rRNA sequences and other phylogenetic markers demonstrate that they belong to the same branch, together with related species within the genus, such as *P. mendocina*, *P. alcaligenes*, *P. pseudoalcaligenes*, and *P. balearica*. Typically, cells are rod shaped, 1 to 3 μm in length and 0.5 μm in width, and have a single polar flagellum. Under certain conditions, one or two lateral flagella with a short wavelength may be produced. Phenotypic traits of the genus include a negative Gram stain, positive catalase and oxidase tests, and a strictly respiratory metabolism. In addition, *P. stutzeri* strains are defined as denitrifiers. They can grow on starch and maltose and have a negative reaction in arginine dihydrolase and glycogen hydrolysis tests. The G+C content of their genomic DNA lies between 60 and 66 mol%. DNA-DNA hybridizations enable at least 17 genomic groups, called genomovars, to be distinguished. Members of the same genomovar have more than 70% similarity in DNA-DNA hybridizations. Members of different genomovars usually have similarity values below 50%.

Differentiation from Other Species

No fluorescent pigments are produced, which differentiates *P. stutzeri* from other members of the fluorescent group of *Pseudomonas* spp. Before the use of genomic approaches to identifying bacteria became widespread, *P. stutzeri* strains were misidentified with other species. This was due to the intrinsic limitations of exclusively phenotypic identification procedures within the former genus *Pseudomonas*. *P. stutzeri* was most commonly confused with other *Pseudomonas* species (*P. mendocina*, *P. pseudoalcaligenes*, *P. putida*); with species actually in other genera (such as *Delftia acidovorans* and *Ralstonia pickettii*); or even with the flavobacteria, *Alcaligenes* or *Achromobacter*. Mandel proposed the species "*Pseudomonas stanieri*" for *P. stutzeri* strains with a low G+C content, around 62% (212); however, G+C content alone is a weak parameter for species differentiation. In some collections, *P. stutzeri* cultures were labeled *P. saccharophila*. The strain OX1 (ATCC BAA-172) was classified phenotypically as a *P. stutzeri* strain (13). It has been intensively studied due to its significant phenotypic characteristics. However, when strain OX1 was characterized taxonomically in detail, it turned out to be a member of the *P. corrugata* phylogenetic branch (73). *Pseudomonas* sp. strain OX1 may be confused phenotypically with *P. stutzeri* because *P. stutzeri* is phenotypically diverse. However, OX1 is genomically distinct.

The species most closely related to *P. stutzeri* is *P. balearica* (formerly genomovar 6 of the species). It shares many basic phenotypic traits with *P. stutzeri* strains and belongs to the same 16S rRNA phylogenetic branch. However, it can be differentiated chemotaxonomically from *P. stutzeri* by its ability to grow above 42°C and by a few other biochemical tests (23).

P. chloritidismutans is a member of genomovar 3. However, it has been proposed as the type strain of a new species (404) and is discussed below (see "Physiological properties"). There is always a danger of drawing taxonomic conclusions from the properties of metabolic systems that are involved in the metabolism of unusual substrates or molecules.

The phylogenies of genes of the *rrn* operon, considered individually or with other housekeeping genes, demonstrate that all *P. stutzeri* strains are monophyletic. Such phylogenetic studies are currently another good tool for discriminating *P. stutzeri* from the rest of the bacterial species. *P. xanthomarina* has recently been described as a new species (289) with only one representative strain. It is located in the same 16S rRNA phylogenetic branch as *P. stutzeri* and *P. balearica*, with sequence similarities above 98%. It can be differentiated phenotypically from both species.

DISCOVERY AND NOMENCLATURAL PROBLEMS

In 1952, C. B. van Niel and M. B. Allen stated in their note on the history of *P. stutzeri*: "During the two decades following the discovery of the denitrification process several notable papers were published on the isolation and characterization of denitrifying bacteria. A study on this literature reveals that Burri and Stutzer (1895) were the first to describe such organisms in sufficient detail to render them recognizable. This applies particularly to their *Bacillus denitrificans II*, an organism of wide distribution and outstanding characteristics, which has been isolated from straw, manure, soil, canal water, etc., and which students of the denitrification process have considered as a very common and easily identifiable denitrifier" (371). The different names that this denitrifier has gained since its discovery are well documented in van Niel and Allen's 1952 publication (371). They include *Bacterium stutzeri* (196), *Bacillus nitrogenes* (229), *Bacillus stutzeri* (68), *Achromobacter sewerinii* (28), *Pseudomonas stutzeri* (322), and *Achromobacter stutzeri* (27). The species "*Pseudomonas stanieri*" was proposed in 1966 by Mandel for those strains with a G+C content of around 62% (212). However, no clear differences in phenotype can be found between *P. stutzeri* and "*Pseudomonas stanieri*." It is not to be confused with *Marinomonas stanieri*, formerly considered a *Pseudomonas* species.

The type strain is Lautrop strain AB 201 (equivalent to Stanier 221, ATCC 17588, CCUG11256, DSM 5190, ICMP 12591, LMG11199, NCIB 11358, and WCPPB 1973). In addition, a reference strain has been proposed for each genomovar (Table 1). Some relevant strains that were previously assigned to other species are *Pseudomonas perfectomarina* strain ZoBell (19), *Alcaligenes faecalis* A15 (380), and *Flavobacterium lutescens* strain ATCC 27951 (24). Many, but not all, strains have been deposited in publicly recognized culture collections, are available for scientific research, and should be used as reference strains.

OCCURRENCE AND ISOLATION PROCEDURES

Detection of *P. stutzeri* basically relies on two methods: (i) enrichment and isolation of pure cultures and (ii) direct analysis without the need for culturing. Both methods are essential to autoecological studies and to understanding the role of the species in the environment.

An elective culture method for the specific enrichment of denitrifiers and the isolation of *P. stutzeri* was developed by Iterson in 1902 (described in 1952 by van Niel and Allen [371]). A mineral medium with 2% nitrate under anaerobic conditions and tartrate (or malate, succinate, malonate, citrate, ethanol, or acetate) leads to a predominant population of *P. stutzeri*, even when some isolates are not able to grow on tartrate in pure culture. Tartrate may be converted anaerobically to an assimilable substrate by other bacteria in the sample. A selection of cells producing colonies with the unusual morphology of *P. stutzeri* permits an efficient isolation procedure from environmental samples. Incubation temperatures of 37°C or above allow a more selective enrichment, which can be combined with denitrifying conditions.

DNA methods based on 16S rRNA sequences have been also designed to detect *P. stutzeri* in DNA extracted directly from environmental samples. Bennasar et al., in 1998, developed PCR primers that were specific to all known genomovars of *P. stutzeri* at that time (24). This served as a confirmation test, as did amplicon cleavage using the restriction enzyme HindIII or a specific DNA probe targeted at the amplified product (24). Amann et al. considered the difficulty of obtaining a DNA probe to cover all of the *P. stutzeri* strains (5). However, they designed a DNA probe for specific 23S rRNA sequences. This is useful in fluorescence in situ hybridization techniques to detect and quantify *P. stutzeri* in environmental samples. Nevertheless, not all strains can be detected, due to the high genetic diversity of the species, including the *rrn* operon.

Besides the *rrn* genes, other genes are now used for functional analysis of ecosystems. These genes also detect *P. stutzeri*. They include *nirS* or *nosZ* for detecting denitrification (46) and *nifH* for analyzing diazotrophic bacteria in the rhizosphere (93). The usefulness of a conserved *nosZ* probe for screening the distribution of denitrifying bacteria with similar N_2O reductases in the environment has been described elsewhere (65, 386). In 2001, Grüntzig et al. developed a very sensitive method based on real-time PCR analysis of DNA isolated from soil and sediment samples (132). However, not all DNAs of the species' strains could be amplified. Specific primers for PCR and an internal probe of the denitrification gene *nirS* enabled less than 100 cells per g of sample to be quantified.

In their analysis of *P. stutzeri* populations in marine waters, Ward and Cockcroft used monoclonal antibodies raised against outer membrane proteins of the strain ZoBell (388). ZoBell originally named this strain "*Pseudomonas perfectomarina*."

Sikorski et al. were able to isolate members of *P. stutzeri* from aquatic habitats and terrestrial ecosystems in a two-step procedure. Firstly, the occurrence of *P. stutzeri* cells was assessed by a previously designed, slightly modified PCR procedure (24, 325). Secondly, the positive samples were screened

TABLE 1. *P. stutzeri* strains cited in the text, with relevant characteristics, origins, and references

Strain	Other designation(s)	Taxonomy[a]	Isolation	Origin, geographic location, and/or physiological characteristic(s)	Reference(s)
CCUG11256	ATCC 17588, DSM5190, LMG11199, Stanier 221	Type strain, gv 1	Pre-1966	Clinical, spinal fluid; Copenhagen, Denmark; siderophore producer	340
ATCC 17591	Stanier 224	Ref, gv 2	1956	Clinical; Copenhagen, Denmark	340
DSM50227	ATCC 11607, LMG1228	Ref, gv 3	Pre-1952	Garden soil; denitrifier	371
19SMN4	DSM6084	Ref, gv 4	1988	Marine sediment; naphthalene degrader; Barcelona, Spain	291
DNSP21	DSM6082	Ref, gv 5	1988	Wastewater; denitrifier; Mallorca, Spain	291
SP1402	DSM6083	Former Ref, gv 6; *P. balearica*	1988	Wastewater; 2-methylnaphthalene degrader; Mallorca, Spain	23
DSM50238	ATCC 17832	Ref, gv 7	Pre-1966	Soil; denitrifier; California	340
JM300	DSM10701	Ref, gv 8	Pre-1980	Soil; California; natural transformation model organism	60
KC	ATCC 55595, DSM7136	Ref, gv 9	1990	Aquifer; California	316
CLN100		Ref, gv 10	1990	Chemical industry wastewater; Germany	114
28a50	CCUG50544, DSM17089	Ref, gv 11	2002	Soil; Tel Aviv airport area, Israel	325
28a39	CCUG50543, DSM17088	Ref, gv 12	2002	Soil; Tel Aviv airport area, Israel	325
28a22	CCUG50542, DSM17087	Ref, gv 13	2002	Soil; Tel Aviv airport area, Israel	325
28a3	CCUG50541, DSM17086	Ref, gv 14	2002	Soil; Tel Aviv airport area, Israel	325
4C29	CCUG50538, DSM17082	Ref, gv 15	2002	Sea sediment; Dangast, Germany	325
24a13	CCUG50539, DSM17083	Ref, gv 16	2002	Soil contaminated with mineral oil; Espelkamp, Germany	325
24a75	CCUG50540, DSM17084	Ref, gv 17	2002	Soil contaminated with mineral oil; Espelkamp, Germany	325
MT-1	CCUG50545, DSM17085	Ref, gv 18	1997	Marine sediment at 11,000-m depth, Mariana Trench	351
1317			1998	Accumulates PHA	141
9A			2003	Alfalfa rhizosphere contaminated with coal tar; Rubinsk, Russia; aromatics degrader; chemotactic	243
A15	LMG10652	gv 1	1981	Rice paddy; nitrogen fixer; Southeast Asia; formerly *Alcaligenes faecalis*	380
A29			2005	Proteolytic	273
AG259				Soil; silver resistant	63
AK61			Pre-1998	Cyanide degrader; metal-plating plant wastewater; Japan	390
AN10		gv 3	1983	Marine sediment; naphthalene degrader; Barcelona, Spain	42, 44
ATCC 14405	ZoBell, CCUG16156	gv 2	1944	Marine; Pacific Ocean, California; formerly *P. perfectomarina*	412, 415
ATCC 17587	Stanier 220, LMG 5838	gv 2	Pre-1966	Clinical; Copenhagen, Denmark	340
ATCC 17589	Stanier 222	gv 1	Pre-1966	Clinical; Copenhagen, Denmark	340
ATCC 17594	Stanier 227	gv 1	Pre-1966	Clinical; Copenhagen, Denmark	340
ATCC 27951		gv 1	1988	Formerly *Flavobacterium lutescens*	24
AW1	DSM13592 ATCC BAA-443	gv 3; *P. chloritidismutans*	2002	Wastewater treatment plant; chlorate reducer	380
BG 2		gv 4	1999	Sulfide-oxidizing bioreactor; thiosulfate oxidizer	337
CFPBD		Pres gv 3	2001	Chlorate reducer	1
ChG 5-2		gv 4	1999	Black Sea (southwest), 120-m depth; thiosulfate oxidizer	337
ChG 5-3		gv 3	1999	Black Sea (south), 120-m depth; thiosulfate oxidizer	337
CMT.9.A	DSM4166		1987	*Sorghum nutans* rhizosphere; nitrogen fixer; Germany	189
JD4		gv 5	1995	Garden soil; denitrifier; Mallorca, Spain	24
JJ			2003	1,2-Dichloroethane-contaminated soil; growth on 2-chloroethanol as denitrifier	95
NF13			1991	Deep-sea hydrothermal vent; sulfur oxidizer; Galapagos rift	300
P16			1994	Creosote-contaminated soil; phenanthrene degrader	348
PDA		Pres gv 1 or 5	2001	Primary digested wastewater on lactate; chlorate reducer	75
PDB		Pres gv 1 or 5	2001	Primary digested wastewater on lactate; chlorate reducer	75

Continued on following page

TABLE 1—*Continued*

Strain	Other designation(s)	Taxonomy[a]	Isolation	Origin, geographic location, and/or physiological characteristic(s)	Reference(s)
PK		Pres gv 3	1999	Soil or sediment; chlorate reducer	75
RC7			1980	Catechol-like siderophore producer	224
RS34			1984	Industrially polluted soil; zinc resistant	135
ST27MN3		gv 4	1988	Marine sediment; naphthalene degrader; Barcelona, Spain	296
WM88			1998	Soil; Illinois; P oxidizer	223
ZP6b			1997	*Capparis spinosa* Rhizosphere; nitrogen fixer; Spain	6

[a] Pres, presumptively; Ref, reference strain; gv, genomovar.

for *P. stutzeri* by means of plating on an artificial seawater medium with ethylene glycol, starch, or maltose as the carbon source under aerobic conditions (325). The characteristic colony morphology of *P. stutzeri* led to a highly efficient isolation procedure: one *P. stutzeri* colony was detected among 9,100 colonies of other bacteria.

However, many strains of *P. stutzeri* that have been studied in detail were isolated by their metabolic peculiarities. They were not specifically isolated for denitrification ability or because *P. stutzeri* was the target of the study.

PHENOTYPIC PROPERTIES

Apart from the 1952 study by van Niel and Allen, the only papers containing detailed descriptions of *P. stutzeri*'s phenotypic properties are those by Stanier et al. in 1966 and Rosselló-Mora et al. in 1994 (295, 340, 371).

Strains of *P. stutzeri*, like most recognized *Pseudomonas* spp., can grow in minimal, chemically defined media, with ammonium ions or nitrate and a single organic molecule as the sole carbon and energy source. No additional growth factors are required. Some *P. stutzeri* strains can grow diazotrophically. This characteristic seems to be rare among the genus *Pseudomonas*. None of the strains tolerate acidic conditions: they do not grow at pH 4.5. *P. stutzeri* has a respiratory metabolism, and oxygen is the terminal electron acceptor. However, all strains can use nitrate as an alternative electron acceptor and can carry out oxygen-repressible denitrification. Denitrification may be delayed or may appear only after serial transfers in nitrate media under semiaerobic conditions (73, 340). Oxidative degradation of aromatic compounds involves the participation of mono- and dioxygenases. Typically, catechol or protocatechuate is the central intermediate in this reaction. Each is cleaved through an *ortho* pathway when no accessory genes are involved in the degradation. Amylolytic activity is one of the phenotypic characteristics of the species. The enzymology of the exo-amylase—which is responsible for the formation of maltotetraose as an end product—has been examined at the molecular level. This enzyme has also been cloned (231). Obradors and Aguilar demonstrated that polyethylene glycol was degraded to yield ethylene glycol, a substrate typically used by *P. stutzeri* strains (241).

The arginine deiminase system ("dihydrolase") catalyzes the conversion of arginine to citrulline and of citrulline to ornithine. It has been used by taxonomists to differentiate species. All *P. stutzeri* strains give a negative test result for this reaction. They also fail to use glycogen and do not liquefy gelatin.

Colony Structures/Types

Colonies can be distinguished by their unusual shape and consistency (Fig. 1). Freshly isolated colonies are adherent, have a characteristic wrinkled appearance, and are reddish brown, not yellow, in color. They are typically hard, dry, and tenaciously coherent. It is easy to remove a colony in its entirety from a solid surface. Colonies generally resemble craters with elevated ridges that often branch and merge, and they have been described as tenacious, with a coral structure. There may be more mucoid protuberances at the periphery than in other areas. The frequent occurrence of irregular polygon-like structures or concentric zones has also been noted (371). The

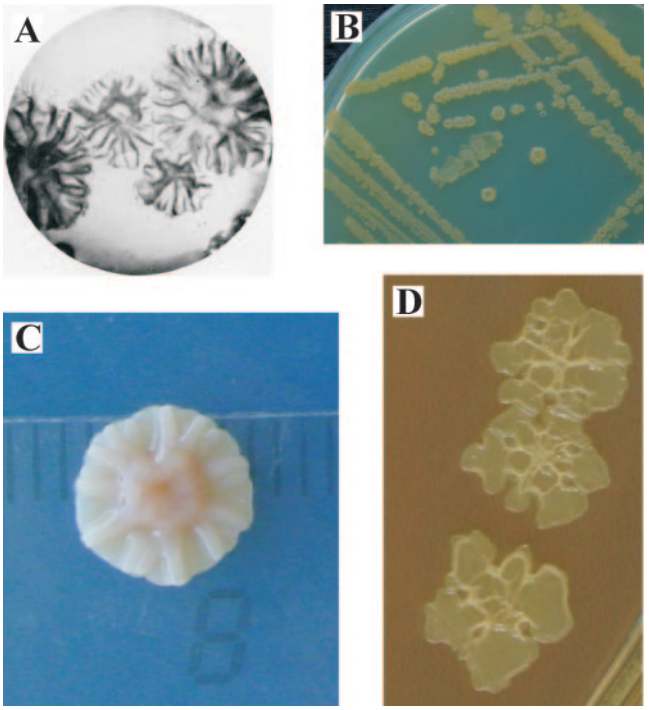

FIG. 1. Colonial morphology. Several typical colonial morphologies of *P. stutzeri* strains. (The image in panel A was taken from reference 371.)

shapes of colonies are neither uniform nor necessarily constant: they change appearance with time. After repeated transfers in laboratory media, colonies may become smooth, butyraceous, and pale in color. This has been described as colonial dissociation. Strain CMT.9.A hydrolyzes agar. This is a rare property and is mainly restricted to marine bacteria. However, the attack may be limited to what is known as "pitting" of the agar (3). Sorokin et al. give a very detailed description of the colonial morphology, differentiating between R-type and S-type colonies (337). The R-type colonies are stable, but the S type produces both colony types under appropriate conditions. Smooth colonies grown on plates at 30°C and stored at 4°C for 24 h often develop a characteristic wrinkled appearance (A. Cladera, personal communication).

P. stutzeri is grouped with the nonpigmented species of the genus, even though many strains' colonies become dark brown. This is due to the high concentration of cytochrome *c* in the cells. No diffusible pigments are produced on agar plates.

Morphological Characterization (Cells, Reserve Materials, Flagella, and Pili) and Chemotaxis

Cells are typically motile and predominantly monotrichous. In some strains, lateral flagella with a short wavelength are also produced. This particularly occurs in young cultures on complex solid media. These lateral flagella could easily be shed during manipulations incidental to flagellar staining (251). It has been suggested that lateral flagella might be involved in the population's swarming or twitching motility on solid surfaces (319). However, type IV pili may also be responsible for this movement. Statistically, the highest number of flagellated cells is reached at the beginning of the exponential growth phase (192). Seventy percent of cells were flagellated in strain AN11: 38% had only one flagellum, and 31% had one or more additional flagella inserted laterally (80).

Caution should be exercised when only phenotypic traits are used for classification. This can clearly be seen in the case of strain ZoBell. This strain (ATCC 14405) was isolated as a marine bacterium and described by ZoBell and Upham as "*Pseudomonas perfectomarinus*" in 1944 (412). Subsequently, this organism became the only member of the species *P. perfectomarina*. Its lack of flagella was emphasized by its assignation to a new species, although the authors who first described this strain stated that it was motile (19, 412). After three passages, enrichment for flagellated bacteria on semisolid tryptone agar enabled a population in which over 80% of cells were flagellated to develop. This revertant strain is motile by means of a single polar flagellum (294).

In a recently published chapter on chemotaxis in *Pseudomonas*, Parales et al. stated, "All *Pseudomonas* species are motile by one or more polar flagella and are highly chemotactic" (258). *P. stutzeri* is no exception. Chemotaxis machinery has not been studied in detail for any *Pseudomonas* species. Moreover, the ranges of attractants or repellents and environmental conditions to which *Pseudomonas* spp. respond remain largely unexplored. They seem to be attracted to virtually all of the organic compounds they can use as growth substrates. However, they are also attracted to other compounds that they are unable to metabolize. Ortega-Calvo et al. studied the chemotactic response of several pseudomonads to polycyclic aromatic hydrocarbon-degrading bacteria (243). Strain 9A of *P. stutzeri* was included in the study. This strain degrades naphthalene, phenanthrene, and anthracene. It was concluded that chemotaxis was positive to naphthalene and to the root exudates of several plants. Chemotaxis may enhance the biodegradation of pollutants in the rhizosphere, at least in laboratory-scale microcosms. Strain KC mineralizes carbon tetrachloride, and motility-enhanced bioremediation in aquifer sediments has been demonstrated (401, 402).

Pseudomonas species have a range of different adhesins that function during initial attachment to a substratum. This leads to biofilm formation. Both flagella and pili seem to be important in the colonization of biotic and abiotic surfaces, particularly in the initial formation of microcolonies. *P. aeruginosa*'s initial biofilm development appears to be conditionally dependent on type IV pili. *P. stutzeri* possesses both flagella and pili but has not been described as a member of consortia that form natural biofilms. Type IV pili confer twitching motility to *P. stutzeri* strains (a bacterial movement based on pilus extension/retraction). This is probably at least partly responsible for many colonies' diffuse borders (J. Sikorski, personal communication). These colonies also correspond to strains that have natural transformation ability.

Chemical Characterization and Chemotaxonomy

DNA base composition. The G+C content of DNA is a useful characteristic in taxonomy for delineating species. It has been proposed that if two strains differ by more than 5% in G+C content, then they should not be allocated to the same species (297). The limit for genus differentiation may be 10%. G+C content in *P. stutzeri* strains has been determined by the thermal denaturation temperature of the DNA and by enzymatically hydrolyzing the DNA and subsequently analyzing it by high-performance liquid chromatography. Reported values vary widely: 60.7 to 66.3 mol% (251) and 60.9 to 65 mol% (291). However, variations are within the accepted limits for members of the same species. The distribution of values was initially considered to be bimodal. This led to the suggestion that *P. stutzeri* might be split into two species (212). Nevertheless, the inclusion of novel strains resulted in a Gaussian distribution.

Protein patterns. Whole-cell protein patterns obtained by denaturing polyacrylamide gel electrophoresis (PAGE) are highly characteristic at the strain level. They have been used for typing and classification purposes (265). *P. stutzeri* strains have been found to be particularly heterogeneous (271, 295). Computer-assisted analysis of the protein bands creates a dendrogram that is in good agreement with the genomovar subdivision of the species (366). This result is not surprising, as whole-cell protein patterns reflect the protein-encoding genes in the whole genome and the genomovars were defined by the similarity values of total DNA-DNA hybridizations.

LPS and immunological characteristics. Lipopolysaccharide (LPS) is the main antigenic molecule on the cell surface. This is considered to be the heat-stable O-antigen of the genus. The specificity of antibodies is related to the composition of the polysaccharide chains projecting outside the cells. Representative *P. stutzeri* strains of the seven known genomovars on which experi-

ments were done showed marked serological diversity. This parallels the LPS O side-chain heterogeneity between strains. In the study by Rosselló et al., antigenic relatedness was observed only between closely related strains of the same genomovar (292).

Outer membrane proteins analyzed by sodium dodecyl sulfate-PAGE gave very similar results for all strains tested, regardless of genomovar ascription. Likewise, similar results were attained for immunoblotting using polyclonal antisera against six representative strains' whole cells. However, a similar procedure, based on Western blotting and immunological fingerprinting of whole-cell proteins using the polyclonal antibody Ab160, raised against *Pseudomonas fluorescens* MT5—called Westprinting (360)—produced a typical protein profile for each strain. Computer-assisted comparisons revealed a distribution in groups that agreed with the strains' genomovar distribution at different similarity levels (25).

Fatty acid composition. Fatty acid composition is a very good taxonomic marker for distinguishing the genus from other genera formerly included in *Pseudomonas* (e.g., *Burkholderia*). These chemotaxonomic characteristics are very useful for identification purposes. Studies of the fatty acid composition of *Pseudomonas* species (158, 246, 341, 367) revealed that the straight-chain saturated fatty acid $C_{16:0}$ and the straight-chain unsaturated fatty acids $C_{16:1}$ and $C_{18:1}$ were the most abundant. These account for 82.3% of total fatty acids in *P. stutzeri*. Minor quantities of the hydroxylated fatty acids 3-OH 10:0 and 3-OH 12:0 were also detected (295). There were no significant differences between genomovars in the other fatty acids. Members of genomovar 6 had a higher content of *cis*-9,10-methylenehexadecanoate (17:0) and *cis*-9,10-methyleneoctadecanoate (19:0). This chemotaxonomic particularity, together with other characteristics, helped to distinguish genomovar 6 as a new species, *Pseudomonas balearica* (23).

Fatty acid composition must be determined under strictly controlled growth conditions, as it is highly dependent on growth substrates. Mrozik et al. describe the changes in fatty acid composition in strains of *P. putida* and *P. stutzeri* during naphthalene degradation (232, 233). The reaction of both strains to the addition of naphthalene was an increase in the saturated/unsaturated ratio and alterations in the percentage of hydroxy, cyclopropane, and branched fatty acids. New fatty acids were detected when the strains were exposed to naphthalene.

Quinone and polyamine composition. The determination of polyamine and quinone composition is a rapid chemotaxonomic identification tool. Putrescine is the main component of all members of the genus *Pseudomonas* (57). Two major polyamines were detected in *P. stutzeri*: putrescine (35.0 to 92.7 μmol/g [dry weight]) and spermidine (8.9 to 29.2 μmol/g [dry weight]). Other polyamines were detected in very small amounts only (1,3-diaminopropane, cadaverine, and spermine) (293). Ubiquinone Q-9 is the only quinone present in all of the *P. stutzeri* strains studied.

PHA. *P. stutzeri* cells do not accumulate polybetahydroxybutyrate. However, the production of novel polyhydroxyalkanoates (PHA) by one strain of the species (strain 1317) has been demonstrated (141). This strain was isolated from oil-contaminated soil in an oil field in northern China. Another *P. stutzeri* strain, YM1006, has been isolated from seawater as a poly(3-hydroxybutyrate)-degrading bacterium, although it does not seem to be able to accumulate this reserve material. The extracellular polybetahydroxybutyrate depolymerase gene ($phaZ_{Pst}$) has been well characterized (242).

Some combinations of unusual phenotypic properties can be very helpful in the preliminary assignment of newly isolated strains to certain species. Alternatively, the absence of one or more of the set's properties suggests that the strain should be excluded from the taxon. For example, in addition to the basic characteristics of a *Pseudomonas* species, the following characteristics strongly suggest that a culture is a strain of *Pseudomonas stutzeri*: denitrification with copious gas emission; the formation of dark, folded, coherent colonies; and the capacity to grow at the expense of starch, maltose, or ethylene glycol. However, in our laboratories we have found that enrichment conditions frequently yield cultures lacking one or more of the key characteristics mentioned above. Such enrichment conditions included the use of aromatic compounds and some of their halogenated derivatives as the sole carbon and energy sources. Although the general phenotypic properties of these cultures could be used a priori as an argument for excluding them from the species, it was surprising to find that some of them were phylogenetically very similar to *P. stutzeri*. This is probably true in the case of a strain ascribed to *Pseudomonas putida* in a patent for the mineralization of halogenated aromatic compounds (U.S. patent no. 4,803,166, 7 February 1989). Its DNA sequences most probably indicate its affiliation to *P. stutzeri*. Detailed analysis of atypical phenotypes (such as the absence of either motility or denitrification) demonstrated in some cases that the characteristic was cryptic and could be expressed when the cells were adapted.

An interesting example of variation to be taken into consideration may be the lack of folded colonies, which, in principle, is taken as an important primary criterion for the isolation. In fact, the discovery of *P. mendocina* at the University of Cuyo, Mendoza, Argentina, was linked to isolations of smooth colonies of *Pseudomonas* which at first were taken to be biovars of *P. stutzeri*.

GENOMIC CHARACTERIZATION AND PHYLOGENY

DNA-DNA Hybridizations

The genomovar concept was originally defined for *P. stutzeri* as a provisional taxonomic status for genotypically similar strains within a bacterial species. Two strains classified phenotypically as members of the *Pseudomonas stutzeri* species were included in the same genomovar when their DNA-DNA similarity values were those generally accepted for members of the same species (more than 70% similarity or less than 5°C difference in thermal denaturation temperature [ΔT_m] values). Members of two different *P. stutzeri* genomovars have 15 to 50% DNA-DNA similarity values or ΔT_m value differences greater than 5°C. Subsequently, this concept has been used taxonomically to group genotypically similar strains in other species, such as *Burkholderia cepacia* and species in the genera *Xanthobacter*, *Azoarcus*, and *Shewanella*, etc. It provides a useful provisional level of classification.

The methods used to calculate DNA-DNA similarity values have differed from one laboratory to another. Palleroni used ^{125}I labeling and/or membrane filters (251). Rosselló et al. used

the ΔT_m method, as described previously (291). Sikorski et al. used the method described by Ziemke et al. (411), with digoxigenin and biotin labeling and quantification of the binding ratio in microtiter plates (327). Vermeiren et al. used DNA-DNA thermal reassociation, measured photometrically (380). The results were consistent with the genomovar subdivision of the species, regardless of the method used to estimate the similarity value.

To date, nine different genomovars have been well documented. Eight new genomovars in the species *P. stutzeri* were put forward recently (327). One reference strain has been proposed for each genomovar and deposited in culture collections. Most strains studied so far are included in genomovar 1 (along with the species' type strain). The genomovars 8 (strain JM300), 9 (strain KC), 10 (strain CLN100), and 18 (strain MT-1) each have only one representative strain. These might be considered genomospecies, sensu Brenner et al. (50). As an example, we can consider strain CLN100, of genomovar 10. It is a representative of a new species from a genomic perspective, sharing many substantial phenotypic and phylogenetic characteristics with members of the *P. stutzeri* phylogenetic branch. Some phenotypic traits can be used to discriminate CLN100 from the *P. stutzeri* and *P. balearica* strains described to date (simultaneous degradation of chloro- and methyl-derivatives of naphthalene and absence of *ortho* cleavage of catechol, etc.). These characteristics could be the basis for describing CLN100 as the type strain of a new species. However, some of these phenotypic traits could be strain specific; therefore, it was preferred not to define a new species until more strains that are genomically and phenotypically similar to strain CLN100 have been described (114).

Genome Size and Organization

Information on genome structure is a very important component of any comprehensive bacterial description. The comparative analysis of bacterial chromosomes on intra- and interspecies levels can provide information about genomic diversity, phylogenetic relationships, and chromosome dynamics. In the genus *Pseudomonas*, genome structure has been studied only for *P. aeruginosa*, *P. fluorescens*, *P. putida*, and *P. stutzeri*. Ginard et al. studied 20 strains of *P. stutzeri* in 1997, representing the seven genomovars known at that time (121). They also studied *P. stutzeri*'s closest relative, *P. balearica*. The genome of *P. stutzeri* strains is made up of one circular chromosome. It ranges from 3.75 to 4.64 Mb in size (20% difference in size). In comparison, *P. aeruginosa* genome sizes, calculated by macrorestriction analysis, range from 6.345 to 6.606 kb, a fluctuation of only about 4%. However, a more recent report on *P. aeruginosa* genome sizes indicates a 20% fluctuation (from 5.2 to 7.1 Mb) (310). The I-CeuI, PacI, and SwaI low-resolution map of *P. stutzeri*'s type strain enabled 12 genes—including four *rrn* operons—and the origin of replication to be located (121). The 20 strains' enzyme digests were used to compare *rrn* backbone organization within the genomovars. The four *rrn* operons seemed to be at similar locations with respect to the origin of replication, as did the rest of the six genes analyzed. In most genomovar reference strains, *rrn* operons are not arranged around the origin of replication but are equally distributed along the chromosome. Large chromosomal rearrangements and differences in genome size seem to be responsible for the differences in genome structure. This suggests that they must have played an important role in *P. stutzeri* diversification and niche colonization. Strains belonging to the same genomovar have similar genome architectures that are well correlated with phylogenetic data (121).

From one to four plasmids were detected in 10 of the 20 strains analyzed in this study (121). The Eckhardt method, using both conventional and pulsed-field gel electrophoresis, turned out to be the most reliable and useful technique for plasmid detection. Seventy-two percent of the plasmids observed were smaller than 50 kb, one plasmid was between 50 and 95 kb, and four plasmids were larger than 95 kb. No two strains shared the same plasmid profile, and no relation was found between genomovars and the distribution of plasmids among the strains. Seven of the 10 plasmid-containing strains were isolated from polluted environments. This is not uncommon in plasmid analyses. A correlation between the degree of contamination and the incidence of plasmid occurrence was found in an environmental study by Baya et al. (20). Naphthalene degradation plasmids are common in *Pseudomonas* spp. However, in eight of the nine naphthalene-degrading strains of *P. stutzeri* studied, the catabolic genes were inserted into an I-CeuI chromosomal fragment, as demonstrated by Southern blot hybridizations with *nahA* and *nahH* probes. The naphthalene genes seem to be plasmid encoded only in strain 19SMN4 (120, 296).

Genotyping

Genotypic intraspecies relationships in *P. stutzeri* strains have been determined by various genotyping methods. These are based on restriction fragment length polymorphism (RFLP) analysis of total DNA, PCR amplification of selected genes, or PCR amplification and restriction analysis. These analytical methods differ in discrimination level between strains. They have been applied simultaneously to all *P. stutzeri* genomovars' reference strains; to *P. balearica*, the strains most closely related to *P. stutzeri*; and to related type strains of the genus *Pseudomonas*. In all methods, computer-assisted analysis generates dendrograms that confirm the consistency of strain clustering with the genomovar subdivisions of the species. Additional typing by multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST) is discussed below.

Methods based on the electrophoretic patterns of macrorestriction fragments (low-frequency restriction fragment analysis) have been used by two independent groups to examine representative strains (121, 271). The restriction enzymes XbaI and SpeI cut the *P. stutzeri* genome of the strains studied into 20 to 48 fragments. These fragments were resolved by pulsed-field gel electrophoresis. They are useful for generating fingerprints, which can be used to explore genome structures and to determine the degree of relatedness of strains. No correlation was found between the similarity of macrorestriction patterns and the subdivision of the species into genomovars. This was due to the high discriminatory power of the two enzymes and the heterogeneity of the restriction patterns. However, some patterns allowed clonal variants between strains to be distinguished. In these cases the related strains belonged to the same

genomovar. The marked heterogeneity was attributed, at least in part, to large chromosomal rearrangements (121).

In the ribotyping procedure, total DNA is purified and then cleaved by restriction endonucleases. Brosch et al. (51) used the enzymes SmaI and HincII in their study of *Pseudomonas* strains. Restriction fragments were separated by electrophoresis, transferred to a nylon membrane, and hybridized with a 16S-23S rRNA probe. Nine strains of *P. stutzeri* clustered together in the dendrogram, which also showed 217 other strains from different *Pseudomonas* species. Two identical bands were detected by HincII in *P. stutzeri*. SmaI profiles were more discriminative, distinguishing from four to eight bands. Members of a single genomovar were grouped in the same branch.

Bennasar et al. (25) revealed genetic diversity and the relationships among *P. stutzeri* strains by rapid molecular typing methods. Repetitive extragenic palindromic PCR and enterobacterial repetitive intergenic consensus PCR analyses, based on DNA consensus sequences, generated fingerprints that were then computer analyzed. Groupings were consistent with the genomic groups that had previously been established by DNA-DNA hybridizations or 16S rRNA sequencing. Members of other *Pseudomonas* species were clearly different. Sikorski et al. (325) carried out random amplified polymorphic DNA (RAPD) PCR analysis in their study of *P. stutzeri* isolates from marine sediments and soils in geographically restricted areas (local populations). The results demonstrated the complex composition and high strain diversity of the local populations studied.

Similar genomic relationships have been revealed by PCR amplification of several genes (16S rRNA, internal transcribed spacer region 1 [ITS1], ITS2, and *rpoB*) and by analyzing the RFLPs generated by several restriction enzymes (25, 133, 325). These methods have confirmed the high genetic diversity of the species, the consistency of genomic groups (genomovars), and the usefulness of the patterns generated for strain identification.

Genetic Diversity: MLEE

Knowledge of the genomic structure of a population is essential to thoroughly understanding a species' characteristics. Such knowledge is particularly important in studies of population dynamics or habitat colonization, as it is used to elucidate genetic exchange in natural populations. The MLEE technique involves determining allozyme variation in a variety of housekeeping enzymes. Codon changes within enzyme genes, leading to amino acid substitutions, are detected electrophoretically by this technique (314). Thus, the variation in chromosomal genes is recorded, and the degree of gene transfer within a species is estimated. This enables relationships between bacterial isolates to be determined and a phylogenetic framework to be constructed.

Two independent research groups have used the MLEE approach in studies of *P. stutzeri* (284, 324). In Sikorski's study, 16 *P. stutzeri* strains belonging to eight different genomovars were analyzed for the allelic profiles of 21 enzymes. A distinctive multilocus genotype was detected in all strains, and up to 11 alleles were detected per locus. In Rius's analysis, 42 *P. stutzeri* strains from nine genomovars (including 9 strains previously studied by Sikorski et al.) and 20 enzymes were studied. The highest number of different alleles found per locus was 32, and all multilocus genotypes were represented by a single strain. Forty-two electrophoretic types were detected. In both analyses, *P. stutzeri* was shown to have a highly polymorphic structure. If both groups' results are combined, 49 different *P. stutzeri* strains have been studied with MLEE. A total of 33 different enzymes were analyzed from these strains. An analysis of this set of 49 strains again demonstrates that all of the multilocus genotypes were represented by a single strain. MLEE studies reveal that *P. stutzeri* is highly polymorphic. The highest genetic diversity described for a species is revealed (284) by an analysis of the members of genomovar 1 only. An analysis of source and place of isolation showed no clear association in clusters. When two subgroups of *P. stutzeri* populations (clinical and environmental isolates) were compared, the mean levels of genetic diversity were not significantly different. This indicates that clinical strains come from the same populations as environmental isolates. This may have important epidemiological implications for the microbiology of *P. stutzeri* infections. However, when two strains were grouped at moderate genetic distances (below 0.55), each pair of strains belonged to the same genomovar.

Genetic Diversity: MLST

MLST has been proposed as a good method for population genetic analysis and for distinguishing clones within a species (98). This method employs the same principles as MLEE, as it detects neutral genetic variation from multiple chromosomal locations. This variation is identified by nucleotide sequence determination of selected loci. Cladera et al. (72) attempted to differentiate *P. stutzeri* populations and to establish the genetic diversity and population structure of the species clearly. They carried out a comparative analysis of gene fragments, using the principles of multilocus sequence analysis. The genes were selected from 26 strains belonging to nine genomovars of the species and from *P. balearica* strains, the species most closely related to *P. stutzeri*. Seven representative chromosomal loci were selected, corresponding to three kinds of genes: (i) housekeeping genes that are universally present in bacteria (16S rRNA and ITS1 region, representing the *rrn* operon, and the *gyrB* and *rpoD* genes, which interact with nucleic acid metabolism, coding for gyrase B and DNA-directed RNA polymerase, respectively) and which have been included in previous *Pseudomonas* taxonomic studies (408); (ii) genes that are characteristic of the species (*catA*, coding for catechol 1,2-dioxygenase, an enzyme responsible for the *ortho* cleavage of catechol in species of RNA group I of *Pseudomonas*, and *nosZ*, nitrous oxide reductase, a metabolically characteristic gene defining this denitrifying species); and (iii) *nahH*, coding for catechol 2,3-dioxygenase, responsible for the *meta* cleavage of catechol, a gene that is considered to be plasmid encoded in the genus *Pseudomonas* but chromosomally encoded in most naphthalene-degrading *P. stutzeri* strains studied to date (296).

All loci were highly polymorphic in the 26 strains studied. The number of nucleotide substitutions per nucleotide site varied from 44.2% for *catA* to 21.8% for *nahH*. The number of alleles varied in the different loci: 4 in *nahH* (16 strains), 18 in *catA* (24 strains), 20 in *gyrB* (26 strains), 17 in *rpoD* (26 strains), 18 in *nosZ* (26 strains), 15 in 16S rRNA (26 strains), and 20 in

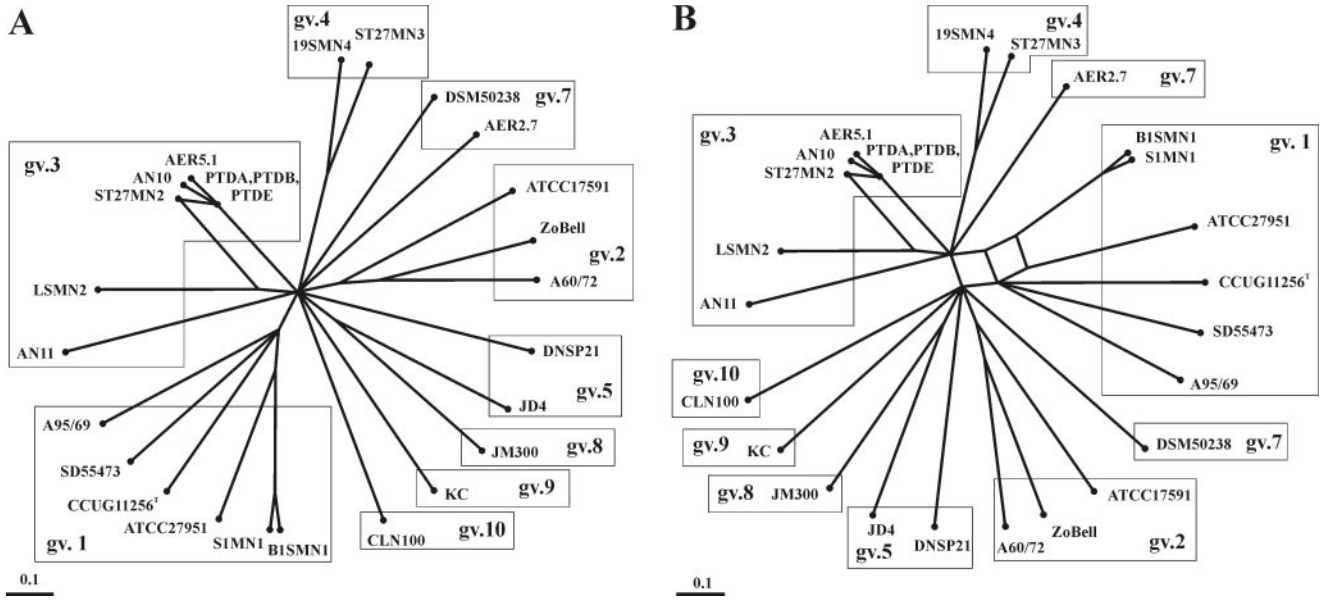

FIG. 2. Split graphs showing the interrelationships of 26 strains of *P. stutzeri* distributed across nine genomovars. (A) The housekeeping genes analyzed (16S rRNA, ITS1, *catA*, *gyrB*, *rpoD*, and *nosZ*) indicate an essentially clonal population structure, with limited recombinational events. (B) When *nahH*, a gene acquired most likely as a consequence of the adaptation of *P. stutzeri* strains to environmental pollutants, is included in the analysis, new branches appear, indicating the transfer of this gene between 13 of the 17 naphthalene-degrading strains studied and the nonstrict clonality of *P. stutzeri*.

ITS1 (26 strains). Apart from *nahH* (a gene that is probably acquired through lateral transfer), the mean number of alleles per locus in the 26 strains was 18.7, an extremely high value. The average number of alleles per locus and strain was 0.72.

In this MLST study (72), the dN/dS ratio—the ratio of nonsynonymous substitutions per nonsynonymous site which resulted in an amino acid replacement (dN) to synonymous substitutions per synonymous site that did not change the amino acid (dS)—was calculated for the genes encoding proteins as a measure of the degree (amount and type) of selection in *P. stutzeri* populations. Changes are selectively neutral when they are independent of the overlying phenotype and the selection pressure dictated by the phenotype's function. The ratio was less than 0.1 in three genes (*gyrB*, *rpoD*, and *nosZ*). The highest dN/dS ratio corresponded to *catA* (0.18). All ratios were much less than 1, indicating that these gene fragments are not under selection. In other words, most of the sequence variability identified is selectively neutral. Synonymous substitutions were at least 5.5 times (1/0.18) more frequent than amino acid changes at any locus.

The number of nucleotide substitutions per nucleotide site was higher than in *Campylobacter jejuni*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Enterobacter faecium*, and species of the *Bacillus cereus* complex. To our knowledge, the number of nucleotide substitutions described for *P. stutzeri* is the highest recorded to date (145). The average numbers of alleles per locus and strain analyzed in the protein-coding genes were 0.72 for *P. stutzeri* (an average of 18.7 alleles per locus in only 26 strains), 0.18 for *C. jejuni*, and 0.43 for the *B. cereus* complex. These values are in good agreement with previous observations made in MLEE studies of most of the strains analyzed by the MLST technique. In such MLEE studies the genetic diversity was the highest described for a species (284). Therefore, the extremely high genetic diversity of the species manifested by MLEE was corroborated by the MLST study.

Figure 2 shows an analysis of the sequence types (STs) identified among 26 independent strains of *P. stutzeri*. This analysis led to the assumption that one different ST per strain can be detected. This is the highest possible number of STs. Remarkably, when two strains had an allele in common they belonged to the same genomovar. There was only one exception: strain JM300 (genomovar 8) has an *rpoD* allele that is identical to strain JD4, one of the two members of genomovar 5. This can be explained by genomovars 5 and 8 having a common ancestor or by a possible lateral gene transfer to JM300, a strain intensively studied due to its natural transformation (206). Another strain, AN10 of genomovar 3, presents a possible recombination event with members of the same genomovar. Strains 19SMN4 and ST27MN3, of genomovar 4, were very closely related in the multilocus sequence analysis. They had identical 16S rRNA, *rpoD*, and *gyrB* genes. Both strains were isolated as naphthalene degraders from samples taken in a wastewater treatment lagoon. However, they were from different habitats (water column and sediment). Molecular typing methods (25, 121, 133) and MLEE (284) had previously demonstrated that both strains were genetically related but different. Again, the enormous genetic diversity of the species was demonstrated in this study. Inclusion of *nahH* in the analysis modifies the topography of the graph, indicating more possible events of lateral gene transfer (Fig. 2).

Phylogeny

Several genes have been used as phylogenetic markers in *P. stutzeri* studies. The most extensively used are the rRNAs, 16S rRNA in particular. However, other genes with different de-

grees of sequence variation have been studied, because they provide useful information for analyzing different phylogenetic levels. Internal transcribed spacer regions ITS1 and ITS2, between the 16S and 23S rRNAs and between the 23S and 5S rRNAs, respectively, in the *rrn* operon present more-variable positions and are most useful in determining close relationships. Recently, Yamamoto et al. (408) studied the sequences of other housekeeping genes (*gyrB* and *rpoD*). These genes are assumed to be less constant than the 16S rRNA molecule among species of the genus *Pseudomonas*. In most cases, the study confirmed the phylogenetic branches that were previously defined by the 16S rRNA sequences in the genus.

Phylogenetic tree reconstructions of the same genes used in the MLST method (16S rRNA, ITS1, *gyrB*, *rpoD*, *nosZ*, and *catA*) were undertaken by Cladera et al. (72). Stability analysis using bootstrap resampling showed that the trees were stable and well defined. Most strains of *P. stutzeri* clustered in the same phylogenetic branch in the gene trees analyzed. They were usually separated from the other closely related species considered, *P. balearica* and *P. mendocina*. Strains belonging to the same genomovar were usually located in the same branch. There were only a few exceptions, which varied depending on the gene analyzed. A consensus phylogenetic tree was constructed for the six genes to deduce a composite molecular phylogeny for *P. stutzeri*. All *P. stutzeri* strains are located in the same phylogenetic branch, and members of each genomovar are clustered together, maintaining the genomovar subdivision of the species. This tree is based on a sequence of no less than 4,551 nucleotides, representing at least 9,546 nucleotides from the respective genomes, as there are four copies of the *rrn* operon in *P. stutzeri*. Therefore, between 0.2 and 0.25% of the chromosome (depending on the strain's genome size) has been compared pairwise in 24 independent isolates.

Clonality

There is enormous genetic diversity in *P. stutzeri*. Despite this, the topologies of the trees and the values of the housekeeping genes' association indices, calculated from MLEE and MLST analyses, indicate that horizontal gene transfer and recombination processes are not enough to disrupt allele associations. This is because there is still a strong linkage disequilibrium among the *P. stutzeri* isolates. These results suggest that the population structure of *P. stutzeri* is strongly clonal, indicating that there is no significant level of recombination through independent assortment that might destroy linkage disequilibrium. Some authors have suggested that recombination events explain some of the diversity found in *P. stutzeri* (324). However, results of studies by Rius et al. (284) and Cladera et al. (72) are clear on this point. They use evidence from linkage disequilibrium analysis to argue strongly against the presence of detectable recombination. In a study on the potential for intraspecific horizontal gene exchange by natural genetic transformation, Lorenz and Sikorski (207) concluded that, with regard to transformation, there is sexual isolation from other *Pseudomonas* species and other genomovars. Gene transfer between genomovars by transformation is limited by sequence divergence at least; heterogamic transformation was reduced in competent cells. The potential to receive genes can also vary greatly among strains. It appears that some strains have a greater potential than others for gene acquisition. It seems that genomovars are free to diverge in neutral sequence characters as a result of sexual isolation mechanisms. These mechanisms prevent randomization of alleles. Nevertheless, the authors consider this border to not be absolute, and foreign sequences may be acquired and fixed.

A careful analysis of some genes, based on incongruences in the phylogenetic trees and/or what is known as relative codon usage, the codon bias index, or the G+C content of the genes, can help to define some metabolic pathways as genes acquired through horizontal transfer. The following examples are considered below: the aromatic degradative pathway, the nitrogenase system, the ability to use chlorate as a terminal electron acceptor, and the energy-yielding reactions in the oxidation of thiosulfate.

TAXONOMIC RANKS: GENOMOVARS

Strains ascribed to the species *P. stutzeri* share some phenotypic traits that distinguish them from other species. In this respect, *P. stutzeri* is a well-defined species that is relatively easy to recognize. However, several intraspecific groups can be delineated genomically and phylogenetically, even when they are monophyletic. In previous polyphasic taxonomic approaches, groups that are phenotypically similar but genotypically different have been referred to as "genospecies," "genomospecies," or "genomic species." A genospecies has been defined in bacteriology as a species that can be discerned only by comparison of nucleic acids. If a specific genospecies cannot be differentiated from another genospecies on the basis of any known phenotypic trait, it should not be named until such a differentiating trait is found (392). Brenner et al. (50) proposed that the term "genospecies" be replaced by "genomospecies." This would avoid confusion with the earlier definition of genospecies, which was a group of strains able to exchange genetic materials. The term "genomic species" is also in use: it is a group of strains with high DNA-DNA hybridization values (76, 297).

Subspecies designations can be used for organisms that are genetically close but phenotypically divergent. In this way, the infraspecific level seems to be phylogenetically valid. It can be distinguished from the infrasubspecific concept of variety. This concept is based solely on selected "utility" attributes that cannot be demonstrated by DNA reassociation (392). Ranks below subspecies are often used to indicate groups of strains that can be distinguished by some special characteristic. Such ranks have no official standing in nomenclature but often have great practical usefulness. An infrasubspecific taxon is one strain or a set of strains that have the same or similar properties and are treated as a taxonomic group.

The "genomovar" concept was coined (291, 363) to clarify the taxonomic status of *P. stutzeri* genomic subgroups. Therefore, the concept was first applied to *P. stutzeri*. It is a useful pragmatic approach to classifying individual strains when they are genomically different from phenotypically closely related strains. It is also of use when phenotypic intragroup variability cannot be clearly established. This occurs when only a small set of strains (or just one) has been isolated. There is no clear phenotypic or biochemical relationship, or a common geographical origin or source of isolation, between members of the

same genomovar in *P. stutzeri*. The suffix "-var" refers to a taxonomic rank below the species level. Nine genomovars (114) have been intensively studied within the species. Members of two different genomovars are genomically distant enough to be considered different genomic species. However, due to the lack of discriminative phenotypic traits, the strains are included in the same nomenspecies. Recent studies undertaken by Sikorski et al. (327) and Romanenko et al. (289) have described some additional *P. stutzeri* isolates that belong to previously described genomovars and others that represent at least eight new genomovars. These results were obtained by 16S rRNA phylogenetic analysis, RAPDs, and DNA-DNA hybridizations (327).

Since its definition, the genomovar concept has been applied to other genomic groups in different bacterial species, such *Burkholderia cepacia* (368) and *Azoarcus* spp. (336). It could be applied to other well-defined genomic groups in species such as *Shewanella putrefaciens* and *Bacillus cereus*, etc. Other authors (e.g., J. P. Euzéby [http://www.bacterio.cict.fr/]) consider "genomovar" to be an unfortunate term, as it assumes that genomic differentiation should be the basis for differentiating bacterial species.

Due to the high genomic diversity of *P. stutzeri* strains, other authors prefer to use supraspecific terms to refer to all of them. Examples are the *P. stutzeri* "group" (337), the *P. stutzeri* "superspecies" (337), and the *P. stutzeri* "complex" (408).

IDENTIFICATION

Phenotypic Identification

Phenotypic identification based on the characteristics given in the species definition and following dichotomous keys is usually satisfactory (26, 115). At present, studies of nutritional properties are frequently carried out with commercial kits designed to reduce the labor involved in traditional methods. Commercial procedures, such as the API 20NE, Microbact NE, and Biolog GN tests, usually identify *P. stutzeri* strains correctly. The identification manuals consider important distinguishing characteristics, such as denitrification or maltose utilization, to not be universal (denitrification is 94% positive, maltose is 69% positive, arginine dihydrolase is 2% positive, and gelatin liquefaction is 1% positive in the API strips). It is assumed that some tests may not be in accordance with the species' typical features. The strain sometimes has to be "adapted" to the test, by growing it under similar, but not strictly selective, conditions prior to the test. Denitrification is a good example of this and is considered below. In a study of the presence and identification of *P. stutzeri* in clinical samples, Holmes (154) stated that routine clinical laboratories have difficulty identifying this species.

A microbial cell expresses some 200 different proteins that can be separated by PAGE. This yields complex banding patterns, which are considered to be highly specific fingerprints (265). Strains with at least 70% DNA similarity tend to have similarities in protein electrophoretograms. Therefore, PAGE is thought to be a sensitive technique for gaining information on the similarities between strains within the same species or subspecies. Individual strains can often be recognized by protein pattern. Under standard growth and PAGE conditions, the patterns are reproducible. Computer-assisted analysis enables the information to be normalized and stored. This method has been used to identify *P. stutzeri* strains when a wide database is available (366).

The Sherlock microbial identification system is based on analyzing total fatty acid profiles. It gives satisfactory results within the genus *Pseudomonas*, including *P. stutzeri*, if the cells are cultured under strictly controlled conditions.

Molecular DNA-Based Identification

A PCR and an oligonucleotide probe method have been developed specifically for detecting and identifying *P. stutzeri*. The amplification primers and the probe were designed from the analysis of available 16S rRNA sequences. Positions that were specific for *P. stutzeri* and differed from the rest of *Pseudomonas* species were selected from variable regions in the *Pseudomonas* 16S rRNA. Positions 743 (G) and 746 (A) fulfilled both criteria, and a 21-nucleotide primer was designed (rps743). A second oligonucleotide, fps158 (17-mer), at positions 142 to 158, was selected as a second specific primer. It produced a 625-bp amplicon in PCR. The specificity of the amplicon was further identified with a DNA probe (17-mer) that included 12 bases of the 5′ end of primer rps743 (25).

A second set of primers, fps158 and rps1271, was developed by Bennasar et al. (24). These primers produced a 1,159-bp amplicon containing a BamHI restriction site. The specificity of the amplicon for *P. stutzeri* was then corroborated by restriction, giving two fragments, of 695 and 465 bp, respectively. A slightly modified set of primers in the same region was used successfully by Sikorski et al. (325).

The three methods permit good molecular differentiation of *P. stutzeri* from other species. They have been used to identify *P. stutzeri* and to detect it in environmental samples, as indicated below (see "Occurrence and Isolation Procedures").

Polyphasic Identification

The species is well-defined phenotypically and chemotaxonomically. However, some of its distinguishing traits are lacking in well-documented strains (starch hydrolysis, arginine dihydrolase activity, and motility, etc.). In addition, many biochemical properties are extremely variable within the species and are not correlated with the genomovar groupings. DNA-DNA similarity values of more than 70% (or less than 5°C difference in thermal denaturation temperatures) are required to definitively assign a strain to a given species. In *P. stutzeri*, a polyphasic taxonomic approach is needed for assigning a new strain to the species: the strain has to agree with the basic phenotypic traits of the species, has to be placed in the same branch as *P. stutzeri* reference strains in the phylogenetic trees of one or more housekeeping genes, and has to show DNA-DNA similarity values of more than 70% with a reference strain of a recognized genomovar. If the last condition is not fulfilled, the strain can be proposed as a new genomovar within the species. If it can be phenotypically distinguished from *P. stutzeri* strains, it can be proposed as a new species.

PHYSIOLOGICAL PROPERTIES

Temperature, Pressure, pH, and O_2 Relationships

The species has a wide range of growth temperatures. Temperatures from 4°C (strain NF13 grew at 4, 22, and 35 but not 55°C [297]) to 45°C (CMT.9.A grows at 45°C) have been cited for individual strains. However, growth at these extreme temperatures seems to be limited to selected strains. Strains that grow at low temperatures are mainly those isolated from cold habitats. Most strains grow at 40°C and 41°C, some at 43°C. The optimum temperature for growth is approximately 35°C. Palleroni et al. (251) subdivided *P. stutzeri* into two biotypes: one clustered around 62% G+C that does not tolerate a temperature of 43°C, and a second of around 65 to 66% G+C that grows at 43°C or higher.

Some strains (NF13, MT-1) have been isolated from the deep-sea bottom. Organisms adapted to the deep-sea environment have to grow under conditions of 2°C and 100-MPa pressure. On 28 February 1996, a sediment sample was obtained from the Mariana Trench by the unmanned submersible Kaiko. It seems likely that this was the first time sediment samples were collected from the world's deepest point without any microbiological contamination from other depths (351). The analysis of amplified 16S rRNA sequences from DNA directly extracted from these sediment samples demonstrated the presence of bacteria belonging to the *P. aeruginosa* branch (Mariana bacteria no. 2 [D87347] and no. 11 [D87346]). Pressure-regulated gene clusters were also amplified. Therefore, in addition to being barotolerant, the bacteria from the Mariana sediment may be barophilic microorganisms. Barophilic microorganisms were isolated by maintaining the conditions of 100 MPa and 4°C. Twenty-eight strains were selected. Strain MT-1, isolate HTA208, was grown on marine agar at 28°C and pH 7.6. Its 16S RNA sequence affiliates the strain with the *P. stutzeri* phylogenetic branch. It was able to grow at a hydrostatic pressure of 30 to 60 MPa, and slight growth occurred at 100 MPa. The growth rate of the *P. stutzeri* type strain was strongly affected by hydrostatic pressure. It must be clarified whether the isolated bacteria are active or inactive under high hydrostatic pressure and low temperature or whether their presence is simply a result of settling of flocculated organic matter.

As mentioned above, no strain tolerates acidic conditions: all fail to grow at pH 4.5. This is probably the reason why there is a negative reaction to the oxidation/fermentation test for the use of carbohydrates. Many *P. stutzeri* strains give a neutral result, as the medium is not buffered and acidification inhibits further growth, even when the strain might be able to use the added sugar.

P. stutzeri strains grow well under atmospheric oxygen. However, microaerophilic conditions have to be established when nitrogen-fixing strains are cultured as diazotrophs. All strains described to date are facultatively anaerobic with nitrate. Some strains are also anaerobic, with chlorate or perchlorate as terminal electron acceptors. Both anaerobic properties are discussed in the following section.

Denitrification

The denitrification process carried out by bacteria makes use of N oxides as terminal electron acceptors for cellular bioenergetics under anaerobic, microaerophilic, and occasionally even aerobic conditions (for reviews, see references 45, 77, 184, 263, and 420). During the denitrification process, which involves a pathway of four successive steps, several metalloproteins catalyze the reduction of nitrate to nitrite, nitric oxide (NO), and finally nitrous oxide (N_2O) to dinitrogen (N_2). The metalloenzymes include nitrate reductase (EC 1.7.99.4), nitrite reductase (EC 1.7.2.1 and EC 1.9.3.2), nitric oxide reductase (EC 1.7.99.7), and nitrous oxide (N_2O) reductase (EC 1.7.99.6) (152).

In contrast to the assimilatory reduction of nitrate or nitrite to ammonia for biosynthetic purposes, denitrification in bacteria is a dissimilatory transformation, associated with energy conservation (420). In other words, the enzymatic electron transfer is coupled to ATP synthesis via proton translocation and the formation of a membrane potential (347). The bacterial process of denitrification is normally a facultative trait. It provides bacteria with a respiratory pathway for anaerobic life (184, 420). The distribution of denitrification capabilities among the prokaryotes does not follow a clear pattern (263). The former genus *Pseudomonas* is one of the largest taxonomic clusters of known denitrifying bacteria. This fact has largely favored the use of species of the genus *Pseudomonas* as model organisms for studying the denitrification process. Within the genus *Pseudomonas*, and probably also within the prokaryotes, much of the relevant work, advances in the biochemical characterization of denitrification, and essential genetics using highly interdisciplinary approaches have been achieved with *P. stutzeri*. Denitrification is a stable trait for *P. stutzeri*; it is one of the most active denitrifying, heterotrophic bacteria, and it has been considered a model system for the denitrification process (420).

Structural gene clusters and the nature of denitrification genes. A total of about 50 genes are needed in a single denitrifying bacterium to encode the denitrification apparatus' core structures (384). The genes contain structural information for the nitrogen oxide reductases and functions for metal processing, cofactor synthesis, electron donation, assembly processes, protein maturation, and regulation. These denitrification genes have a chromosomal location in *P. stutzeri*.

The complete denitrification process that leads to N_2 formation starts with nitrate reduction. Therefore, many bacteria have more than one of the three types of nitrate reductases: soluble assimilatory nitrate reductase and two dissimilatory reductases that are further subdivided into respiratory and periplasmic nitrate reductase (230). In *P. stutzeri*, the genes coding for the dissimilatory reductases, the *nar* genes, are not linked to the denitrification genes sensu stricto (which include the *nir-nor* loci and the *nos* genes; see below). Instead, they form a separate locus in this bacterium (48, 420).

As in other denitrifying bacteria that depend on the cytochrome cd_1 nitrite reductase, in *P. stutzeri* the genes encoding functions for nitrite respiration (*nir*) and nitric oxide (NO) respiration (*nor*) seem to be preferentially organized in a mixed cluster made up of both types of genes (8, 48, 88, 169, 170, 384, 414). Effectively, this is a single denitrification supercluster of about 30 kb. It contains 33 genes and was located and mapped on a 56-kb BamHI fragment in *P. stutzeri* (strain ZoBell) (48). In addition to the *nir-nor* genes, this cluster harbors the *nos* genes, which are needed for the respiratory reduction of

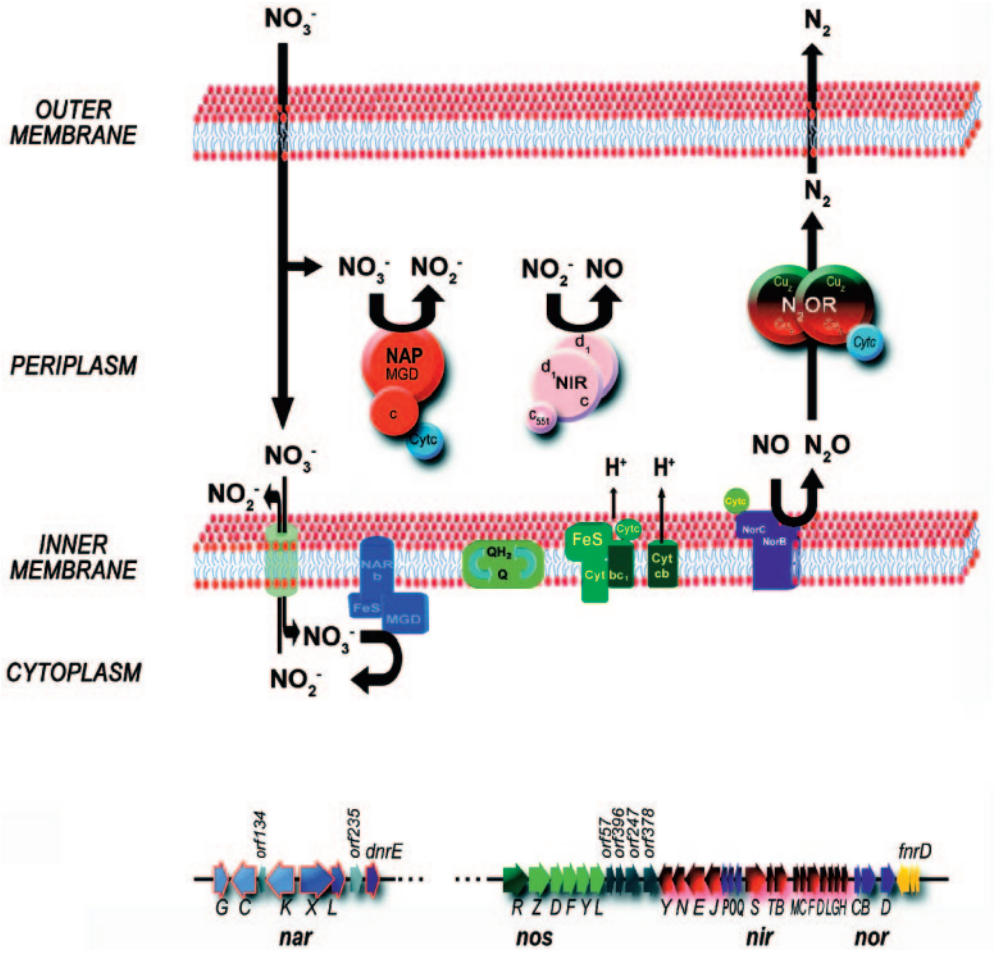

FIG. 3. Denitrification process. Shown is a hypothetical model of the denitrification pathway in *P. stutzeri*, which comprises respiratory nitrate reductase (NAR), nitrite reductase (NIR), NO reductase (NOR), and N_2O reductase (N_2OR). Furthermore, evidence for the presence of a periplasmic nitrate reductase (NAP) in *P. stutzeri* exists (420). The postulated nitrate/nitrite antiporter at the inner membrane has been also included in the model. The organization of the *nar* (137), *nir*, *nor*, and *nos* (420) genes of *P. stutzeri* is also shown. The numbers of amino acids of the hypothetical gene products are used to label unassigned ORFs. Abbreviations: *b*, heme B; *c*, heme C; c_{551}, cytochrome c_{551}; Cu_A and Cu_Z, copper centers A and Z, respectively; Cyt *c*, unspecified *c*-type cytochromes accepting electrons from bc_1; Cyt *cb*, cytochrome *cb* terminal oxidase complex; Cyt bc_1, cytochrome bc_1 complex; d_1, heme D_1; FeS, iron-sulfur centers; MGD, molybdenum cofactor; *orf*, open reading frames; Q, QH_2 quinone cycle. Gene abbreviations are described in the text. (Adapted from references 138 and 230; adapted from reference 420 with permission.)

nitrous oxide (N_2O) and are about 14 kb from the *nir* genes. Figure 3 shows that the gene organization in *P. stutzeri* subclusters is *nos-nir-nor*. Twenty-three of the 33 genes recognized in the *P. stutzeri* denitrification cluster are transcribed in the same direction. Two groups of seven (*nirO*, *nirP*, *nirQ*, *nirJEN*, and *nirY*) and three (*fnrD* and its two adjacent open reading frames [ORFs]) genes are transcribed in the opposite direction. At least 10 transcriptional units have been clearly defined with confidence. However, the definitive number remains open, since not all of the promoters have been mapped (420). Furthermore, polycistronic transcripts for *norCB* and *nirSTB* in *P. stutzeri* have been identified experimentally (418, 420).

(i) *nar* genes. The organization of the genes coding for respiratory nitrate reduction (*nar*) in *P. stutzeri* has been almost completely determined (14, 137). Some of the *nar* genes have homologs in the *Escherichia coli* nitrate respiration apparatus. These include *narK* (encoding a putative nitrite transporter/facilitator permease), *narG*, and *narXL*. The 5′ end of *narL* overlaps the 3′ end of an ORF. Other characteristic *E. coli* genes that are either missing or unidentified in *P. stutzeri* are *narH*, *narJ*, and *narI*. An additional gene (*orf134*) that, together with *narK*, encodes a hypothetical transporter similar to fungal or plant nitrate transporters (*narC*) is missing in *E. coli*. An additional ORF immediately follows the *narL* gene. A sequence encoding an FNR family transcription factor, DnrE (137), follows directly from this ORF. Both the regulatory and the structural genes have opposite orientations in *P. stutzeri* (137).

Moreover, homologs of the structural genes encoding the subunit NapA (a second dissimilatory nitrate reductase in the form of a periplasmic, dissimilatory-type enzyme found in many denitrifiers, such as *Cupriavidus necator* H16) have been detected by hybridizing a 109-kb SpeI fragment from *P. stutzeri* genomic DNA. These loci are probably not linked to the *P. stutzeri* 30-kb denitrification cluster (384). Additionally, a partial *napA* gene for periplasmic nitrate reduc-

tase has been detected by PCR in the *P. stutzeri* strain ZoBell (239). The amplified fragment (380 nucleotides) has 85% identity on a 223-nucleotide stretch of the *Cupriavidus necator* H16 megaplasmid, encoding key enzymes for H_2-based lithoautotrophy and anaerobiosis. The features in this part of the sequence belong to a large subunit of the periplasmic nitrate reductase. Similarly, when a shorter fragment (92 nucleotides) from the complete genome of *Erwinia carotovora* subsp. *atroseptica* SCRI1043 is used, the same fragment has 86% identity with the features that are described for this part of the *Erwinia carotovora* genome sequence as a periplasmic nitrate reductase.

(ii) *nir* genes. The nitrite reductase gene sequence (cytochrome cd_1), *nirS*, in two strains of *P. stutzeri* has been determined. The cytochrome cd_1 from *P. stutzeri* ZoBell was isolated by a phage expression library, and JM300 was isolated with protein-derived oligonucleotide probes (170, 332). In addition to the *nirS* gene, the *nir* gene subcluster contains *nirTBM* genes. The *nirT* gene encodes tetraheme cytochrome, and *nirB* encodes diheme cytochrome (cytochrome c_{552}). Tetraheme cytochrome encoded by *nirT* may have a putative electron donor function (170). However, it seems that in *P. stutzeri*, *nirM* encodes the electron donor (cytochrome c_{551}) for NirS. The *nirD* locus has been established by Tn*5* mutagenesis. The function of its gene product(s) may be related to processing of cytochrome cd_1 or to heme D_1 biosynthesis (169, 413).

In addition, the *nirS* gene is part of clusters that harbor genes for the biosynthesis of heme D_1, a cofactor of denitrifiers. The cytochrome cd_1 nitrite reductase depends on this cofactor. Such genes seem to be distributed over two loci in *P. stutzeri*: *nirJEN* and *nirCFDLGH* (123, 254). As in other *Pseudomonas* spp., several genes, e.g., *nirDLGH*, appear to be duplicated in *P. stutzeri* (254). The gene *nirE*, found in *P. stutzeri* upstream of *nirS* (transcribed in the opposite direction and part of the cluster *nirQJENY* [384]), encodes a putative methyltransferase for the heme D_1 pathway. Two *P. stutzeri* ORFs, *orf393* and *orf507*, are homologous to the *nirJ* and *nirN* genes of *P. aeruginosa*, respectively (123). The gene product of *nirN* (*orf507*) seems to affect anaerobic growth and in vivo nitrite reduction (123). The *nirF* gene is preceded in the *nir* clusters by *nirC* (formerly orf5), which probably encodes a putatively periplasmic monoheme cytochrome *c*. The presumed function of this cytochrome is related to the maturation of NirS (170). Both *nirE* and the putative *nirMCFDLGH* operons have recognition motifs for the anaerobic regulator FNR in their promoter regions.

A putative LysR-type regulator has been located in an ORF (*nirY*) found between the *nos* and *nir* operons in *P. stutzeri* (123). The LysR-type regulator belongs to a family of factors involved in the control of a wide variety of processes, including oxygen stress regulation (309).

Finally, *P. stutzeri* has a *nirR* gene encoding a 25.6-kDa protein that affects nitrite reduction, i.e., NirS synthesis (169). This *nirR* gene is not located in the described denitrification gene cluster and has no significant similarity to known proteins.

(iii) *nor* genes. Generally, two groups of NO reductases (NORs) can be distinguished from a comparison of primary structures: the so-called short-chain NORs (scNORs) (450 amino acids) and the long-chain NORs (lcNORs) (about 760 amino acids). Furthermore, this division corresponds to electron donor specificities: scNORs are part of a cytochrome *c* complex, whereas lcNORs derive electrons from quinol. Thus, these two enzyme forms have been named "cNOR" and "qNOR," respectively, in some studies (146). The genes encoding the NO reductase (cytochrome *bc*-type complex), *norCB*, were the first scNOR genes to be identified. They were established by reverse genetics on purified NOR from *P. stutzeri* (47, 48). A cosmid library of *P. stutzeri* was mapped by using oligonucleotide probes derived from the N terminus of the purified NorC subunit (47).

The *nor* genes in *P. stutzeri* are arranged in three transcriptional units that consist of a *norCB* operon, a *nirQOP* operon, and a monocistronic *norD* transcript. The *dnrN* operon follows *norD* downstream. It includes (encoded on the cDNA strand) a gene for the *nor* regulator, DnrD (385). The *norC* gene product functions as a cytochrome *c* subunit of scNORs. Immediately downstream from *norC* is an ORF (*norB*) that encodes a strongly hydrophobic protein. In fact, the *norB* gene encodes the catalytic subunit of the scNOR and oxidizes cytochrome *c*. Evidence that this ORF represents the structural gene of the cytochrome *b* subunit came from a deletion-replacement mutation of this region. This rendered the Nor^- *P. stutzeri* strain MK321 immunonegative for an antiserum against cytochrome *b* (47). The *norD* gene is downstream of *norCB*. This gene presumably encodes a cytoplasmic protein that affects the expression and function of both NirS and NorCB.

The *P. stutzeri* accessory genes *nirQOP* are situated upstream from *norCB* and are separated by approximately 7 kb of the *nir* genes, encoding the cytochrome cd_1 nitrite reductase and the components for heme D_1 biosynthesis. The *nirQ* gene (*norQ*, formerly *orf8*) is positioned immediately upstream of *nirS*. The *nirQ* gene has a certain sequence similarity to regulators included in the NtrC family (its product has been predicted to be a potential denitrification regulatory component), and it is transcribed in an orientation opposite that of *nirS* (48). Both nitrite and NO respiratory reduction processes are affected by the mutagenesis of *nirQ* (it affects the catalytic functions of NirS and NorCB). This shows that there is a dependency between these processes (171).

The *nirO* gene (formerly *orf175*) encodes a five-span membrane protein that affects the yield and rate of anaerobic growth. It is similar to the cytochrome *c* oxidase subunit III. Finally, *nirP* (*norF*, formerly *orf82*) encodes a two- or three-span membrane protein and is involved in NO and nitrite reduction.

(iv) *nos* genes. The *nos* gene subcluster is located 9 kb upstream of the *nir* cluster. *P. stutzeri nos* genes are needed for the anaerobic respiration of nitrous oxide. This is the final part of the whole denitrification process. The *nos*-encoding region is approximately 8 kb and contains *nosZ* (the structural gene for the copper-containing enzyme nitrous oxide reductase), genes for copper chromophore biosynthesis, and a supposed regulatory region (382, 414).

The *nosR* gene, located upstream from *nosZ*, encodes a membrane-bound transacting regulatory component that is necessary for the transcription of *nosZ* (84–86, 416). The codon usage for NosR shows the characteristics of a typical *Pseudomonas* gene. There is a high overall G+C content (62.4

mol%) and a preference for G or C at the third codon position (84). Furthermore, inverted repeats at the end of *nosR* are not prominent (84).

The synthesis of the functional multicopper enzyme nitrous oxide reductase (N_2OR) needs an assembly apparatus and a maturation process to insert the prosthetic copper (155, 414). To achieve this, *P. stutzeri* has some genes immediately downstream of the N_2OR structural gene. They encode several of the accessory proteins required in the biosynthesis of the catalytically active enzyme. Three maturation genes, *nosDFY*, encode the corresponding proteins. These products are involved in acquiring or processing copper to form a catalytically active N_2O reductase (155, 414). The process includes the formation of a putative ABC transporter complex (consisting of NosD, NosF, and NosY) that extends to both sides of the cytoplasmic membrane (155). Sequence similarity leads to the deduction that *nosF* may encode an ATP/GTPase (155, 414). Moreover, the expressed and purified NosF protein has a structural similarity to the ATPase of maltose or histidine ABC transporters. If any of the *nosDYF* maturation genes are mutationally inactivated, the CuZ center is missing in the resulting enzyme (155, 274, 420).

In addition, the *nos* operon encodes a Cu chaperone, NosL. The predicted presequence of the gene product derived from *nosL* (formerly *orf4*) is similar to that of lipoproteins (48). Furthermore, like many other bacteria, *P. stutzeri* has a Tat (twin-arginine) (143, 305) translocation system for exporting proteins (in addition to the Sec system), which seem to be transported in a folded form (30, 285, 405). Downstream of *nosL*, the *nos* region contains information that encodes a component of the Tat translocon, TatE (155). It is of note that the *P. stutzeri tatE* locus (formerly identified as *orf57*) is unlinked to the rest of the *tat* genes (123, 143).

Finally, the *nnrS* operon is situated immediately downstream of the *nos* operon. The *nnrS* operon includes a gene (*orf396*) that encodes a putative heme-Cu protein (NnrS) and a member of the short-chain dehydrogenase family (Orf247) (155). Additionally, in this region the *orf378* gene codes for a putative membrane-bound protein (Orf378).

Metalloenzymes involved in the denitrification process. (i) Nitrate respiration and NaRs. Respiratory nitrate reductases (NaRs) are complexes that have either two or three subunits, depending on the isolation method. *Pseudomonas stutzeri* strain ZoBell (ATCC 14405) has a membrane-bound nitrate reductase (EC 1.7.99.4) containing three subunits ($\alpha\beta\gamma$). It can be prepared by detergent extraction (148). The heterotrimeric enzyme has the electronic spectrum and absorbance intensity of a diheme protein. Nitrate reductase contains, per M_r 172,000, about 13 iron-sulfur groups and one atom of molybdenum bound to a pterin cofactor (39). In *P. stutzeri* dissimilatory nitrate reductases, the organic moiety of the molybdenum cofactor is a molybdopterin guanine dinucleotide (109). The name "bactopterin" was initially proposed for the modified bacterial Mo cofactor (226). The cofactor in the molybdoenzymes is thought to modulate the redox behavior of the metal. It also aids in electron transfer to or from other redox centers without the pterin cofactor and undergoes a redox process itself (149). The pterin unit connects the electron flow between the molybdenum and the iron-sulfur center (148). Furthermore, the cofactor has a structural function: it fixes the molybdenum core in the center of the protein matrix (94, 148).

In addition, a two-subunit ($\alpha\beta$) form of dissimilatory nitrate reductase can be separated from the membrane-residing γ subunit by a heat solubilization step. The $\alpha\beta$ unit alone has the same catalytic center as $\alpha\beta\gamma$, the α subunit (NarG), which consists of molybdenum and two pterin cofactors. The ligand environment of molybdenum in the active center seems to be unaltered by heat treatment of $\alpha\beta$ and $\alpha\beta\gamma$ preparations, as the electron paramagnetic resonance spectrum properties for the catalytically active molybdenum center signal, Mo(V), are almost identical in both cases (39, 148).

The iron-sulfur complexes in the β subunit (NarH) seem to participate in electron transport from the membrane's quinol pool. The small γ subunit (NarI), which spans the membrane, is a cytochrome *b* protein that contains two *b*-type heme groups (148). The topology of the γ subunit was predicted (by means of an analogy with the NarI subunit from *E. coli* [89]) to be a transmembrane anchor which holds a two-subunit ($\alpha\beta$) form on the cytoplasmic side of the membrane (29). NarI is attributed with quinol oxidation and electron transport to the β subunit.

The purified, soluble, $\alpha\beta$ form of the enzyme from *P. stutzeri* has been seen to have high specific activity (71 U/mg, when one unit of nitrate reductase activity is defined as the production of 1 μmol nitrite per min). The enzyme ($\alpha\beta$-NaR) has a pH range for optimum activity of 7.5 to 8.0, regardless of the NaCl concentration (1 mM to 1 M). NaR activity is strongly dependent on temperature. The maximum temperature is 76°C. The enzyme is competitively inhibited by azide but not by chlorate (no inhibition was found in concentrations of up to 100 mM $NaClO_3$) (148). The $\alpha\beta$-NaR form of the enzyme has K_m values of 3.2 to 3.8 mM for nitrate (148). However, the K_m affinity of the *P. stutzeri* $\alpha\beta\gamma$ nitrate reductase, determined with exogenous redox mediators, was 0.49 ± 0.07 mM at saturating methyl viologen concentrations (41). These differences could be explained by the fact that electron donors first have to reduce the γ subunit in the holoform. In contrast, electron donors have easier access to the iron-sulfur centers in the $\alpha\beta$ form. As a result, the $\alpha\beta\gamma$ form reaches substrate saturation at a lower nitrate concentration (148).

Many denitrifiers have a second dissimilatory nitrate reductase, in the form of a periplasmic dissimilatory-type enzyme (e.g., *napA* in *C. necator* H16). There was no initial evidence for such reductases in the denitrifying *Pseudomonas* species. However, sequence- and hybridization-based analyses have demonstrated their presence in *P. stutzeri* (see above). In general, these periplasmic dissimilatory-type enzymes have a NapA subunit that binds a molybdenum cofactor. They may also have a four-cysteine motif near the N terminus, to attach a 4Fe-4S cluster. In addition, a small-subunit NapB with two potential heme C-binding sites was detected in the sequence. This seems to be needed by these dissimilatory nitrate reductases. Furthermore, a NapC protein belonging to a homologous family of tetraheme *c*-type cytochromes was first reported as *P. stutzeri* NirT (170, 420). The putative role of NapC involves electron transfer between a quinol and the periplasmic nitrate reductase. The physiological role of this periplasmic dissimilatory nitrate reductase could be to promote the transition from aerobiosis to anaerobiosis (147, 240). Whereas membrane-bound respiratory nitrate reductase is expressed

only under anaerobic growth conditions, periplasmic nitrate reductase is synthesized and active in the presence of oxygen (21, 321). In addition, both enzymes are under nitrate control, exerted via the sensor protein NarX or NarQ.

(ii) Properties of NarL and NarX proteins. NarL of *P. stutzeri* is a 218-amino-acid protein, M_r 24,378. It has 51 and 47% positional identity with the *E. coli* proteins NarL and NarP, respectively (137). NarL regulates the *narG* operon. It acts at the transcriptional level by activating the *narG* operon. However, it does not activate the other structural genes of oxidoreductases involved in denitrification (137). Several of its residues (Asp13, Asp14, Asp59, and Lys109) correspond to a set of conserved amino acids found in response regulator proteins. In particular, the aspartic acid residues form an acidic pocket which is part of the phosphoryl acceptor chemistry (185, 236, 259, 343).

The *P. stutzeri*-derived protein NarX consists of 648 amino acids, M_r 71,791. It has 31% positional identity with NarX and NarQ of *E. coli*. Hydropathy and transmembrane prediction analysis seem to suggest that it has two membrane-spanning regions. A carboxy-terminal cytoplasmic domain (C) and an internal periplasmic domain (also known as the P box) have been delimited (137). The C domain has characteristic conserved regions (termed H, N, and D) for the histidine protein kinase family (344) and is therefore a common feature of sensor proteins. It is thought to be important in conferring specificity on sensor response regulator interaction (259). Both the periplasmic P and the cytoplasmic C regions (a stretch of conserved residues intercalated between the aforementioned H and N regions) are conserved in either nitrate- or nitrite-responsive sensory kinases and are specific for NarX-type sensor proteins (137). NarX functions as a sensory component that has an approximately twofold preference for nitrate, i.e., nitrite is about half as active an inducer as nitrate (399). The P region is responsible both for binding nitrate and nitrite and for harboring the essential elements for distinguishing between these ions (137).

(iii) Nitrite respiration and NiRs. Nitrite reductases (NiRs) are key periplasmic enzymes in denitrification. They are responsible for catalyzing the first step of a process that leads to the formation of a gaseous intermediate, which is no longer available to most living organisms. The nitrite reductase reaction in denitrifying bacteria is usually performed by the activity of two metalloenzymes. These enzymes are different in terms of their structure and prosthetic metal compounds. The metalloenzymes are the cytochrome cd_1 (which contains the hemes *c* and d_1 as essential cofactors and is encoded by *nirS*) (83) and a copper-containing nitrite reductase at the active site (which is encoded by the *nirK* gene) (420). The two nitrite reductases have never been found within the same cell. Neither of them could be exclusively associated with a particular member of the *Proteobacteria* (416).

The *nirS* gene in *P. stutzeri* (186) encodes the 62-kDa subunit of the homodimeric cytochrome cd_1 nitrite reductase (EC 1.9.3.2) (393). *P. stutzeri* mutants that have lost cytochrome cd_1 can no longer utilize nitrite (413). The enzyme has a quaternary structure α_2 and a molecular mass of 119 to 134 kDa. The prosthetic groups are heme C and heme D_1. Both of these groups are present in each subunit to render cytochrome cd_1, a tetraheme protein. Heme D_1 is noncovalently bound and is extractable from the enzyme by acidified acetone. Reinsertion of heme D_1 leads to restored activity and to spectroscopic properties of the protein (393). Heme D_1 of cytochrome cd_1 consists of an unusual macrocycle with a set of oxo, methyl, and acrylate substituents. This has been proven by chemical synthesis (228, 406). Cytochrome cd_1 catalyzes the reduction of nitrite to NO through the oxidation of cytochrome c_{551} (electron donor) (420). Its activity level with nitrite is 4.15 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (420).

The biogenesis of the periplasmic protein cytochrome cd_1 involves translocation of the protein across the membrane. This is accompanied by the simultaneous or separate transport of prosthetic groups into the periplasm, covalent binding of heme C and insertion of the noncovalent heme D_1, and finally folding of the protein into its mature form. The translocation of cytochrome cd_1 to the periplasm proceeds in the absence of heme D_1 (123, 413) and, probably as it occurs in other microorganisms, heme C (248, 249). Heme attachment occurs on the periplasmic side of the membrane (420).

Heme C biosynthesis in *P. stutzeri* proceeds via the glutamate (C-5) pathway (214). The central metabolite is glutamyl-$tRNA^{Glu}$, formed by glutamyl-tRNA synthetase (EC 6.1.1.17) (162). A functional denitrification apparatus is dependent on the expression of genes for heme D_1 biosynthesis. It is assumed that NirE catalyzes the methylation of uroporphyrinogen III during heme D_1 synthesis, yielding precorrin-2. Dehydrogenation of this intermediate gives sirohydrochlorin. Despite being homologous to CysG, NirE of *P. stutzeri* (123) needs an N-terminal domain to catalyze dehydrogenation. In addition, Fe chelation in the siroheme pathway is missing. The conversion of precorrin-2 to heme D_1 is probably catalyzed by *nirD* locus products (NirCFDLGH). Indeed, mutations in each particular gene of this locus result either in the absence of heme D_1 from the enzyme or in a nonfunctional cytochrome cd_1 (254, 413).

NirJ and the *orf393* gene product have a certain similarity to the PqqE/PqqIII/Pqq proteins, which have unknown functions in the biosynthetic pathway of pyrroloquinoline quinone; NifB is involved in nitrogenase Mo cofactor biosynthesis. The N-terminal region of these proteins has a conserved cysteine motif: CXXXCXYC. This motif is different in NirJ: CXXXC XXCY. Moreover, the C-terminal domain of NirJ is rich in cysteines. Therefore, a metal-binding site has been postulated. An additional protein, NirN (formerly the *orf507* product), has an overall similarity to NirF and NirS, and its heme C-binding domain is comparable to that of NirC (123).

Finally, several of the *nir* gene products involved in biosynthetic or auxiliary functions have potential export signals, which indicates that periplasmic compartmentalization takes part in the final maturation steps of NirS.

(iv) Nitric oxide respiration and NORs. The respiratory reduction of nitric oxide (NO) is part of a biogeochemical process sustained by prokaryotes. NO is an essential substrate for nitrate and nitrite denitrifiers that release nitrous oxide or dinitrogen as products. Thus, NO is of bioenergetic importance to denitrifying bacteria as both a respiratory substrate and an electron acceptor in anaerobic environments. The NORs are integral membrane proteins. They are responsible for the reduction of NO to N_2O and are members of the heme-copper oxidase superfamily.

The search for NORs in *P. stutzeri* led to the identification of

the first purified and biochemically characterized enzyme, in the form of a two-subunit cytochrome *bc* complex (144). The hypothesis that NORs and heme-copper oxidases (369, 418, 420) have a common ancestor was based on the following evolutionary considerations: the reasonable association of oxygen (aerobic respiration) and nitrate respiration (bacterial denitrification) and the relationship between nitrous oxide reductases and cytochrome *c* oxidases (421), proposed on the basis of unexpected structural similarities between the *P. stutzeri* NOR (NorB sequence) and the cbb_3 terminal oxidase. The high sequence similarity of the catalytic subunits of NOR and cbb_3 oxidases has enabled an accurate three-dimensional structural model of the transmembrane helix and cofactor arrangement of NorB to be constructed. This was achieved by combining the amino acid sequence of the NorB protein from *P. stutzeri* with crystallographic data from *Paracoccus denitrificans* oxidase (161, 173). The proposed three-dimensional model of the membrane topology is characterized by a core catalytic subunit spanning the membrane 12 times (227, 417, 418). The NOR catalytic subunit is a binuclear center (high and low spin). The high-spin center contains a heme iron and a second nonheme metal, which is Fe in NOR and Cu in oxidases. The NOR nonheme Fe is referred to as FeB, which is clearly analogous to the oxidase CuB. The presence of this second metal (Cu or Fe) is a prerequisite for the catalytic activity of both NOR and O_2 reductases (122, 173). The second metal center is a six-coordinate low-spin heme. It acts as an electron transfer center between the donor and the binuclear center. The catalytic subunit of NOR has six topologically conserved histidine residues, for coordinating the two heme groups and a nonheme Fe (161). The resulting NorB model is a compact hydrophobic molecule that limits the exposure of polar surface areas on either side of the membrane.

The structural organization of the *P. stutzeri* NOR genes indicates that there is a single 2.2-kb transcript from the *norCB* operon, rendering a two-subunit core complex (418). In fact, when NOR from *P. stutzeri* is purified, a two-subunit composition and the results of activity assays showed that a minimal composition of two subunits is sufficient for catalysis (91, 176).

The absorption spectrum shows the characteristic spectral features of both heme *c* and heme *b* (421). The isolated NorB subunit, in its reduced form, has absorption maxima at 428, 531, and 560 nm. The isolated, reduced NorC subunit has absorption maxima at 418, 523, and 551.5 nm. NorC is a membrane-bound, monoheme, *c*-type cytochrome. Its N terminus is oriented toward the cytoplasm, and it has a large heme *c*-binding domain of 120 amino acids residing in the periplasm. A single sequence motif, CXXCH, for covalent heme *c* attachment is located in the periplasmic domain. This motif follows the single transmembrane helix for anchoring the protein in the membrane (418, 421). The exposure of the heme *c*-binding domain toward the periplasm was shown by a topologically sensitive reporter gene fusion into Leu67. This was carried out in the immediate vicinity of the heme attachment site, 61-CIGCH-65, of *P. stutzeri* NOR (173). The orientation of NorC toward the outside allows this protein to interact with a periplasmic cytochrome or (pseudo)azurin and supply electrons to the membrane-bound NorB.

(v) Nitrous oxide respiration and N_2ORs. Nitrous oxide reduction is the final step in the denitrification pathway and is catalyzed by the enzyme N_2OR. The gene encoding N_2OR (*nosZ*) is largely unique to denitrifying bacteria. The diversity of *nosZ* has been used to detect denitrifier-specific DNA in environmental samples (307, 308).

N_2OR from *P. stutzeri* has probably been more intensively studied than N_2ORs from other species (420). It is a periplasmic dimer enzyme that exists in several forms. These forms are distinguished by their redox and spectroscopic properties. Form I can be isolated anaerobically in high-activity "purple" species. What is known as form II ("pink" species) is obtained when N_2OR is purified under aerobic conditions. Form II has low activity and a low Cu content, presumably due to oxygen affecting the catalytic center (283, 420). Form I is converted to the blue form III when dithionite is added. Form IV can be prepared from the apoenzyme by incubation with Cu(II). This form is catalytically inactive. Finally, form V of N_2OR carries only Cu_A. It was obtained from the *P. stutzeri* mutant MK402, which is unable to create the catalytic center (283, 419).

Each subunit of the enzyme, which is encoded by *nosZ*, contains two binuclear copper centers. The metal ion content is at least six Cu ions per subunit. The two binuclear centers are termed Cu_A, the entry site for electrons, and Cu_Z, the substrate-binding site (4). The model of the Cu_A center is well described. Its properties are unique among proteins' Cu centers. It is thought to be restricted for cytochrome *c* oxidase (COX) by a mononuclear $Cu(Cys)_2(His)_2$ structure (for a detailed review, see reference 420). In the model, certain conserved histidine residues are involved in the mature protein's copper-binding activity (153, 420). The histidines at positions 583 and 626 coordinate with two cysteines (at positions 618 and 622) and a methionine (at position 629) to bind the copper atoms in *P. stutzeri* (308).

Regarding the Cu_Z, or catalytic, center, eight conserved histidine residues that are likely to be involved in the coordination of copper in the protein have been observed (416). The issue of whether Cu ligation is carried out by amino acids other than histidine is crucial to explaining the N_2OR catalytic site (99, 106). It seems that only one region in the amino acid sequence has the spacing and sulfur-containing amino acid side chains needed to coordinate the additional binuclear copper center (308). For this reason, the region between amino acids 132 and 178 has been proposed as the catalytic, or Cu_Z, site. This site contains four of the eight conserved histidines (308).

Chlorate and Perchlorate as Terminal Electron Acceptors

For over 50 years, it has been known that bacteria can reduce chlorate (ClO_3^-) and perchlorate (ClO_4^-) under anaerobic conditions. Many nitrate-reducing bacteria in pure cultures reduce chlorate and perchlorate [which is usually referred to as (per)chlorate] by means of membrane-bound respiratory nitrate reductases and assimilatory nitrate reductases. In all cases, chlorite (ClO_2^-) is produced as a toxic end product. For many years, there was no evidence that these bacteria could grow using this metabolism. It is now known that specialized bacteria that can grow by anaerobic reductive dissimilation of (per)chlorate into innocuous chloride have evolved. For a recent review, see the work of Coates and Achenbach (75). All known dissimilatory (per)chlorate-reducing

bacteria (DCRB) are facultatively anaerobic or microaerophilic bacteria. Some, but not all, are able to respire nitrate. The DCRB are phylogenetically diverse. Some isolates belong to the *Gammaproteobacteria*. The strains PK, CFPBD, PDA, and PDB have been affiliated with the *P. stutzeri* phylogenetic branch by 16S rRNA sequence analysis. They also seem to be affiliated with genomovars 3 and 1 or 5 in the phylogenetic trees. Strain AW1 is a member of *P. stutzeri* genomovar 3. Wolterink et al. have proposed this strain as the type and only member of a new species, *P. chloritidismutans* (404). Chlorate and perchlorate reduction specifically involves a *c*-type cytochrome in the transfer of electrons to (per)chlorate. A periplasmic oxygen-sensitive perchlorate reductase has been characterized recently (179). In addition to perchlorate, this reductase also reduces chlorate, nitrate, iodate, and bromate. The next biochemical step is the quantitative dismutation of chlorite into chloride and O_2 by chlorite dismutase. Chlorite dismutase is a highly specific enzyme that does not act on other analogous anions. Studies with a chlorite dismutase-specific immunoprobe indicated that this enzyme is present on the outer membrane of all DPRB and that it is highly conserved among these organisms, regardless of their phylogenetic affiliation. Expression of the chlorite dismutase gene (*cld*) is constitutive in strains PDA and PK. Genomic organization has been studied in strain PK: a gene encoding a *c*-type cytochrome lies between *cld* and a transposase gene. The transposase gene is followed by the chlorate reductase operon, with the gene order *clrABDC* (α subunit, β subunit, chaperone protein, and γ subunit). A recent comparison of a phylogenetic tree based on 16S rRNA with a tree developed from the *cld* gene sequences of 11 diverse DPRB demonstrated significant discrepancies. The results of this comparison supported evolution through horizontal gene transfer (22). As mentioned above, strain AW1 is a member of genomovar 3. The definition of this strain as a new species (*P. chloritidismutans*) was based only on its ability to use chlorate, rather than nitrate, as the terminal electron acceptor. However, careful adaptation to nitrate use enabled a denitrifying variant to be isolated. The only difference between this new species and *P. stutzeri* is its ability to dechlorinate (74). This characteristic is most probably acquired through horizontal gene transfer. This case demonstrates the danger of drawing taxonomic conclusions from homologies that are found in metabolic systems involved in the use of unusual substrates.

Organic Compounds Used as the Sole Carbon and Energy Source

Extensive nutritional studies of carbon substrates (more than 150) used by *P. stutzeri* strains have been carried out by Stanier et al (340). Conventional methodologies and commercial kits were later used by other investigators (131, 295). These showed that intraspecies heterogeneity was high but *P. stutzeri* strains clustered separately from other phenons in the *P. aeruginosa* group, as pointed out by Palleroni and Doudoroff in their review on the genus *Pseudomonas* (253a).

In an exhaustive phenotypic study, Rosselló-Mora et al. (295) analyzed a total of 327 biochemical characteristics in 48 strains. The expanded physiological analysis did not improve the situation that was previously observed in the 102-characteristic study (291). This high degree of intraspecies physiological heterogeneity had already been observed by Palleroni and coworkers and by others (113, 251, 340, 371) and in other later numerical studies (115, 291). Numerical analysis of the data did not lead to genomovar-specific clusters but reflected considerable heterogeneity within genomovars. Characterization of individual genomic groups on the basis of biochemical tests was not possible.

All strains tested were positive for the following activities with the indicated substrates: growth on gluconate, D-glucose, D-maltose, starch, glycerol, acetate, butyrate, isobutyrate, isovalerate, propionate, fumarate, glutarate, glycolate, glyoxylate, DL-3-hydroxybutyrate, itaconate, DL-lactate, DL-malate, malonate, oxaloacetate, 2-oxoglutarate, pyruvate, succinate, D-alanine, D-asparagine, L-glutamate, L-glutamine, L-isoleucine, and L-proline and hydrolysis of L-alanine-*para*-nitroanilide. One hundred seven characteristics were variable in the 48 strains tested. The 48 phenotypic tests enabling the best discrimination between genomic groups of *Pseudomonas stutzeri* were selected. They are listed in the original publication by Rosselló-Mora and coworkers (295).

The mineralization, or cometabolism, of xenobiotics or anthropogenic substrates by specialized strains merits special attention. It is described in "Biodegradation and useful properties for biotechnological applications," below.

Inorganic Energy Sources (Thiosulfate)

The phylogenetic diversity of sulfur- and thiosulfate-oxidizing bacteria has been of interest to several authors. The evolution of such bacteria can be clarified by molecular investigation of the genes encoding these pathways' enzymes, together with an analysis of the 16S rRNA phylogenies of bacteria that have these properties. Strain NF13 was isolated by Ruby and coworkers from the Galapagos rift hydrothermal vents (190, 300). It was assumed that H_2S could be the predominant energy source for chemosynthesis in this habitat. Enrichment cultures under selective conditions for sulfur-oxidizing bacteria yielded several physiological groups of strains. One group, represented by strain NF13, was able to grow heterotrophically in media containing peptone or yeast extract either with or without thiosulfate. Acid was produced when thiosulfate was present. This group was unable to utilize CO_2 as the sole source of carbon. Of all the sulfur-oxidizing bacteria isolated, only strain NF13 was found to fix nitrogen. Strain NF13 was studied phylogenetically by Lane and coworkers using the 16S rRNA sequence (190). It was classified as an unnamed "thiobacillus" within the *Gammaproteobacteria*. Recent analysis (A. Cladera, personal communication) clearly placed isolate NF13 in the *P. stutzeri* phylogenetic branch. The phenotypic properties analyzed by Ruby and coworkers (300) were in accordance with ascription to the species: gram-negative, denitrifier, motile, nonfermentative.

More recently, Sorokin and coworkers analyzed the anaerobic oxidation of thiosulfate to tetrathionate by obligately heterotrophic bacteria (337). Strains were isolated from seawater and freshwater and from a sulfide-oxidizing bioreactor. All isolates were obligately heterotrophic. In phenotypic and genomic identification analyses, they were classified as members of *P. stutzeri* (seven strains) and *P. balearica* (one strain).

Of the seven *P. stutzeri* isolates, four were ascribed by DNA-DNA hybridizations to genomovars 3, 4, and 5. The remaining isolates seemed to cluster in another genomic group. Strain ATCC 27951, of "*Flavobacterium lutescens*," was included in this study (it had previously been reclassified as *P. stutzeri* by Bennasar et al. [25]). All seven strains oxidized thiosulfate to tetrathionate using nitrite, nitrate, or N_2O as an electron acceptor. Thiosulfate oxidation under anaerobic conditions was much slower than in the presence of oxygen. In addition, it was controlled by the availability of an organic electron donor. The oxidation of thiosulfate to tetrathionate (yielding one electron) instead of sulfate (yielding eight electrons) does not generate enough energy to support autotrophic growth (a high-energy-requiring process). It is therefore not surprising that tetrathionate-forming isolates are obligate heterotrophs (337). They grow under these circumstances as chemolithoheterotrophs, as the provision of thiosulfate increased their growth yield in acetate-limited continuous cultures.

Sijderius, in 1946, studied some *P. stutzeri* strains with this ability in his dissertation, "Heterotrophe Bacterien, die Thiosulfaat oxydereeren" (322). Whether this chemolithotrophy is restricted to specialized strains or is a general property of the species has not yet been studied. All strains studied were isolated from sulfur-rich environments, with the exception of strain ATCC 27951, which was isolated (surprisingly) from a yogurt in Algeria.

Production of Siderophores

Pyoverdines are important pigments from a taxonomic and physiological perspective, as they function as efficient siderophores. Their production is enhanced under conditions of iron starvation. Although they are not pigmented, some strains of *P. stutzeri* synthesize siderophores. *P. stutzeri* ATCC 17588 produces desferriferrioxamines E and D2 (224, 225). A different strain (RC7) produces a catechol-like siderophore (63). No siderophores have been detected in *P. stutzeri* YPL-1 (202). Siderotyping is also a powerful technique for discriminating species within the genus *Pseudomonas* (225). It is interesting to note that the internal heterogeneity of the nonfluorescent species *P. stutzeri* is also reflected in its siderophore production capacity.

The best studied siderophore is produced by strain KC, the reference strain and only member of genomovar 9 (316). It is highly likely that this is a secondary siderophore in this strain (200). *P. stutzeri* KC can degrade carbon tetrachloride (CT) (trade names, Freon 10 and Halon 104) to carbon dioxide, chloride ions, and other nonvolatile compounds, such as formate. Chloroform is not formed in this process. CT is used in the manufacture of fluorocarbon propellants, as a cleaner and degreasing solvent, and as a fumigant. The EPA estimates that 10% of U.S. groundwater may be contaminated with CT. CT is a toxic, carcinogenic, and ozone-depleting xenobiotic compound detected in groundwater. For CT transformation to occur, *P. stutzeri* KC must be grown in an anaerobic, slightly alkaline medium at around pH 8. The high pH lowers iron solubility, limiting the iron concentration of the growth medium. The medium must also contain nitrate, an electron donor (such as acetate), and trace levels of copper. Strain KC secretes the molecule PDTC (pyridine-2,6-bisthiocarboxylate), which has a siderophore function. PDTC has been implicated in the uptake of other transition metals in addition to iron (200). It is believed to be an iron chelator that is fortuitously involved in CT transformation. Because of its ability to effectively scavenge iron, strain KC has a competitive advantage over bacteria that lack this ability. The iron chelator alone may transform CT. This is indicated by the fact that the supernatant alone (containing the chelator) from washed cells transforms CT. In its active form and in the presence of copper, PDTC transforms CT to carbon dioxide and other nonvolatile products, including formate and chloride ions.

A *pdt* locus corresponding to the PDTC biosynthetic pathway in the strain KC genome has been mapped and cloned as a 25,746-bp insert in the pT31 cosmid (199, 200, 315). The *pdt* operon was sequenced, and a low-density PDTC microarray consisting of 17 PCR-amplified ORFs was printed on chemically modified glass substrates. This was the first published attempt to apply microarray technology to the parallel detection of multiple target genes in *P. stutzeri* for environmental monitoring (235).

Nitrogen Fixation

After years of controversy, it now seems that several strains that are unambiguously classified as true *Pseudomonas* species can be added to the list of nitrogen fixers, on the basis of physiological properties, nitrogenase assays, phylogenetic studies, and the detection of *nifH* by hybridization or PCR amplification and sequencing (66). All well-classified nitrogen-fixing *Pseudomonas* strains described in the literature are members of *P. stutzeri* (92). Former *Pseudomonas* strains from other species able to fix N_2 were transferred to other genera in the α- and β-*Proteobacteria* ("*Pseudomonas paucimobilis*," "*P. diazotrophicus*," and "*P. saccharophila*," etc.). *P. stutzeri*'s simultaneous capacity for nitrogen fixation and denitrification may be of relevance to overall nitrogen cycling in several ecosystems. *P. azotofigens* has only one strain ($6H33b^T$) and has been described recently (140) as a novel nitrogen-fixing species isolated from a compost pile.

Pseudomonas stutzeri A15 is a member of genomovar 1. It is a particularly predominant diazotrophic strain. It was isolated from the rice paddy rhizosphere and is widely used as a rice inoculant in China (136, 270, 380, 409). Previously identified as an *Alcaligenes faecalis* strain, it has been widely studied physiologically, biochemically, and genomically. Strain A15 is able to colonize and infect rice roots and to grow endophytically (92). It may provide rice plants with fixed nitrogen and hence promote plant growth. Another strain, *P. stutzeri* CMT.9.A, was isolated from the roots of a *Sorghum nutans* cultivar in Germany (189). It has not been assigned to any known genomovar. *P. stutzeri* JM300, the only member of genomovar 8, is a denitrifying soil isolate (18, 60). Strain ZP6b, classified as *P. stutzeri* "var. *mendocina*," was isolated from the rhizosphere of capers (*Capparis spinosa*) in Spain (6). Functional nitrogenase activity was tested by the acetylene reduction assay and by the incorporation of ^{15}N. Rates of acetylene reduction in the nitrogenase assay are in the range of those reported for other strains that are presently considered to be *Pseudomonas* species (66). Nitrogen fixation occurs at low oxygen tension under microaerobic conditions, as has been observed in many aerobic

diazotrophs. The optimal pO_2 for nitrogen fixation by strain CMT.9.A is 0.01 atm. In addition to nitrogen-fixing ability, some *P. stutzeri* strains, such as JM300 and CMT.9.A, show hydrogenase activity (18), supplying additional energy for metabolism.

The *nifH* gene, encoding the nitrogenase protein, is considered to be the diagnostic gene for nitrogen fixation. It has been detected in all diazotrophic *P. stutzeri* strains studied to date. A *nifHDK* probe from *Klebsiella pneumoniae* gave a positive signal against the EcoRI-restricted total DNA of strain ZP6b (6). Both *nifH* and *nifDK* probes from *Azospirillum brasilense* also gave a positive signal against strain A1501 (a derivative of strain A15). Partial *nifH* sequences from strains A15 and CMT.9.A are identical. The *nifH* phylogeny in the domain *Bacteria* is largely congruent with phylogenetic trees based on 16S rRNA. They are within the cluster of other strains of the *Gammaproteobacteria* (380). Some strains have been analyzed for the presence of alternative nitrogenase systems. Genomic DNA from strains CMT.9.A and JM300 did not hybridize with a *nifDK* probe encoding the large subunit of Mo-nitrogenase in *Azotobacter chroococcum* (105). In addition, no signal was detected with the genes of the alternative nitrogenases *anfDGK* and *vnfDGK*. The other known pseudomonad that fixes nitrogen, *P. azotofigens* 6H33bT, has *nifH* and *nifD* genes that are closely related to the corresponding genes of strain A15.

Deduced amino acid sequences for the *P. stutzeri* A15 *nifHDK* operon had the highest identity (87 to 91%) with the respective *A. vinelandii* homologs (92). Moreover, the gene organization in the *nifH* region of *P. stutzeri* A15 was identical to the gene organization of *A. vinelandii*. The close relationship between the species has been revealed by a detailed phylogenetic analysis of *A. vinelandii*. This led to the suggestion that *Azotobacter* could be considered a *Pseudomonas* in disguise (277).

The regulation of *nif* genes has been studied only for strain A1501. This strain is similar to A15 (if not identical). Strain A1501 was reisolated from rice roots inoculated with strain A15. Results suggest that *P. stutzeri* has a large number of NtrC family response regulators, as does *P. aeruginosa*. Differences were detected in a *P. stutzeri* NtrB mutant that had impaired nitrogen fixation. No such differences were found in the case of *Azotobacter* spp. Moreover, NifA controls NtrBC expression. This may reflect the fact that the regulatory circuit is different from other organisms. The on/off switch of nitrogenase activity and the role of the *nif* genes also reflect interesting features of this particular strain. A study to identify which genes are switched on during rice colonization and switched off during free-living growth on a synthetic medium has been conducted by Rediers et al. (276). Some of the corresponding genes are involved in stress response, chemotaxis metabolism, and global regulation. Others encode putative proteins that have either unknown functions or no significant homology to known proteins.

A 50-kb plasmid that may carry some genetic information for nitrogen fixation in strain CMT.9.A has been identified. When this plasmid is cured, there is an associated loss of N_2 fixation capability (189). No plasmid was detected in strain A1501. This is consistent with the assumption that *nif* genes have a chromosomal localization in strain A1501. The origin of the nitrogen-fixing genes in some strains of *P. stutzeri* can be explained by a plausible lateral gene transfer acquisition. This hypothesis seems to be supported by *nifH* phylogenies (380).

Phosphite and Hypophosphite Oxidation

A number of bacteria have been shown to be capable of oxidizing reduced phosphorous compounds when these are provided as the sole source of phosphorous. Inorganic phosphite and hypophosphite can be used as such a source. They are oxidized to phosphate by several species, including members of the genus *Pseudomonas*. In a screening of bacteria that oxidize reduced phosphorous compounds to phosphate, Metcalf and Wolfe, in 1998, were able to isolate 10 bacterial strains after enrichment under selective conditions (223). Strain WM88 was studied in detail. It is a *P. stutzeri* strain that is closely related to the genomovar 3 reference strain DSM50227. Related collection strains, including four known *P. stutzeri* strains, were not able to oxidize hypophosphite. However, strain DSM50227 (a clinical isolate) was able to oxidize phosphite. The genes required by strain WM88 have been cloned and studied, together with the respective enzymes. Two operons in the Pho regulon (*htx* and *ptx*) are needed to use phosphite and hypophosphite as alternative P sources (395). An *Alcaligenes faecalis* strain was isolated recently. This strain has *htx* genes that are virtually identical to their homologs in *P. stutzeri*, indicating that horizontal gene transfer may have occurred. However, *pdtx* in *A. faecalis* is different from the homolog in *P. stutzeri* (400). Whether the *htx* genes are widely distributed in *P. stutzeri* or restricted to strain WM88 remains to be studied.

Biodegradation and Useful Properties for Biotechnological Applications

Pseudomonas stutzeri is a ubiquitous bacterium with a high degree of physiological and genetic adaptability. It is present in a large number of different natural environments (see "Habitats and ecological relevance," below). Like other *Pseudomonas* species (e.g., *P. putida*), *P. stutzeri* is involved in environmentally important metabolic activities. Some of its major tasks are metal cycling and degradation of biogenic and xenobiotic compounds (oil derivatives—aromatic and nonaromatic hydrocarbons—and biocides).

Metal cycling. Although metals are essential nutrients, they can be toxic in excess. Moreover, some metals are toxic and have no beneficial purposes. As a result, bacteria have developed systems to ensure the availability of essential metals and, simultaneously, to handle metal toxicity. *P. stutzeri* is no exception. Three distinct types of siderophores (nocardamine, an arginine conjugate of 2,3-dihydroxybenzoic acid, and pyridine-2,6-dithiocarboxylic acid) have been described for this species. These siderophores ensure the availability of essential metals, such as cobalt, copper, iron, and nickel (63, 64, 224, 313, 345). Furthermore, several *P. stutzeri* strains have been described due to their high biosorption potential and resistance to metals such as aluminum (422), chromium (12, 172), cobalt (172), copper (69, 215), germanium (370), lead (215), manganese (172), nickel (272, 346), plutonium (255), selenium (157), silver (135), thallium (172), titanium (38), uranium (172), vanadium

(172), and zinc (34, 172, 215).

Nearly all metal resistance systems seem to be gene encoded, and they are found on plasmids in most cases (328). However, most reported *P. stutzeri* strains' resistance mechanisms remain poorly understood. In fact, the only well-characterized systems are the mercury resistance mechanisms carried on plasmid pPB of "*P. stutzeri*" OX1 (16, 280, 281). As mentioned above, this strain does not belong to the *P. stutzeri* species (see "Definition of the species and differentiation from other *Pseudomonas* species," above). Two distinct *P. stutzeri* strains have generated enormous biological and biotechnological interest: strain AG259 and strain RS34.

P. stutzeri AG259 is a silver-resistant strain isolated from the soil of a silver mine in Utah (135). Its silver resistance mechanism is still poorly understood, but it seems to be gene encoded on the plasmid pKK1 (135, 362). Although plasmid pKK1 is still unsequenced, it has been demonstrated that the encoded silver resistance mechanism is energy dependent (329). This mechanism seems to produce intracellular silver-sulfide complexes (331). Interestingly, *P. stutzeri* AG259 is also able to accumulate large amounts of germanium, copper, lead, and zinc on the cells by energy-independent passive binding (215, 330). In contrast, a more recent report (183) shows that *P. stutzeri* AG259 accumulates silver-based single crystals in the periplasm. This suggests that silver resistance in *P. stutzeri* AG259 involves metal efflux and metal binding. This is also the case in other well-characterized bacterial silver resistance systems (134, 201). The size of the periplasmic silver-based crystals (up to 200 nm) and their well-defined compositions and shapes (equilateral triangles and hexagons) suggest that they have great potential as organic-metal composites in thin-film and surface-coating technology (183).

P. stutzeri RS34 is a zinc-resistant strain isolated from an industrially polluted soil in New Delhi, India (34). This strain efficiently accumulates large amounts of zinc on its outer membrane through morphological and ultrastructural changes (36). Its zinc resistance mechanism is still unknown and does not resemble any known mechanisms (70). However, its use in removing zinc from solutions, low-grade ores, and ore tailings has been demonstrated (35, 37).

An exhaustive study of nickel-resistant bacteria from anthropogenically nickel-polluted and naturally nickel-percolated ecosystems has been undertaken (346). This study analyzed a *P. stutzeri* strain isolated from a soil sample from New Caledonia. The sample was taken from the rhizosphere of *Sebertia acuminata*, a plant that hyperaccumulates nickel in its latex (25%) and leaves (1%). After anaerobic enrichment, the strain was isolated as a denitrifier in the presence of 10 mM $NiCl_2$. *P. stutzeri* was resistant to 3 mM Ni, as well as to Co, Zn, and Cu. Nickel resistance genes were detected by Southern blotting and DNA-DNA hybridization with DNA probes. These genes seem to be located in a plasmid detected by the Kado and Liu method.

Crude oil, oil derivatives, and aliphatic hydrocarbons. Although *P. stutzeri* was one of the first alkane-degrading microorganisms identified—it was identified as *Bacterium stutzeri* (333)—few reports of crude oil-, oil derivative-, and/or aliphatic hydrocarbon-degrading *P. stutzeri* strains have appeared in the literature (81, 95, 156, 163, 166, 268). In contrast, much information is available for other *Pseudomonas* species, such as *P. aeruginosa*, *P. fluorescens*, *P. oleovorans*, and *P. putida* (reviewed in reference 365). Nevertheless, one study directly isolated (with no prior enrichment) and identified 297 gasoline-degrading bacteria from a contaminated aquifer (282). A strong predominance by *Pseudomonas* spp. was observed (86.9% of all strains). *P. stutzeri* was the third most frequently isolated *Pseudomonas* species in this study (7.4% of all strains, 10.2% of *Pseudomonas* strains). In addition, a cultivation-independent analysis (based on 16S rRNA amplification and sequence) of the dynamics of bacterial communities was carried out during a field-scale evaluation of bioremediation on a mudflat beach contaminated with buried oil (288). This study demonstrated that *P. stutzeri* and *Alcanivorax borkumensis* are key microorganisms in dissipating hydrocarbon pollution on maritime beaches. These results also show that enrichment methods may be biased toward the isolation of non-*P. stutzeri* *Pseudomonas* spp.

In spite of the small number of aliphatic hydrocarbon-degrading *P. stutzeri* isolates, two distinct *P. stutzeri* strains are of biological and biotechnological interest: strain KC and strain JJ.

As mentioned above, *P. stutzeri* strain KC is an aquifer isolate that transforms CT to carbon dioxide, formate, and other nonvolatile products. This process occurs only under anoxic conditions, and no chloroform is formed (81, 101, 198, 355). CT has been found as a pollutant in soils and groundwater (159). It can also be mineralized biotechnologically. The fast transformation of CT by strain KC has enabled it to be used in bioaugmentation strategies. In such strategies it forms a biocurtain for the in situ remediation of a CT-contaminated aquifer (102, 401, 402).

P. stutzeri strain JJ was isolated from 1,2-dichloroethane-contaminated soil (95). It is the first microorganism known to grow anaerobically on 2-chloroethanol under denitrifying conditions. Strain JJ requires anoxic conditions for 2-chloroethanol degradation. However, it has been suggested that the 2-chlorocatechol degradation pathway in this denitrifying strain is the same as that found in aerobic bacteria (96). In industry, 2-chloroethanol is used mainly in the synthesis of insecticides and as a solvent (95). It is metabolized by mammalian alcohol dehydrogenase to 2-chloroacetaldehyde, which is considered to be mutagenic (217). Very little is known about the emission and fate of 2-chloroethanol in the environment, as this compound is not included in routine soil pollution analyses. However, the interest in strain JJ and its 2-chloroethanol anoxygenic degradation capability resides in the fact that (i) many soils contaminated with chlorinated aliphatics are anoxic and (ii) nitrate is often present in groundwater (79, 116). Therefore, strain JJ may be of use in 2-chloroethanol bioremediation (95).

Aromatic hydrocarbons. The benzene ring is one of the most widely distributed chemical structures in nature, as it appears in the recycling process of plant-derived material (139). In addition, its persistence in the environment is induced by its thermodynamic stability (87). Aromatic compounds are considered to be major environmental pollutants. Benzene and four of its relatives [polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), benzo(A)pyrene, and benzo(B)fluoranthene] have been in the top 10 of the National Priority List of Hazardous Substances since 1997 (http://www

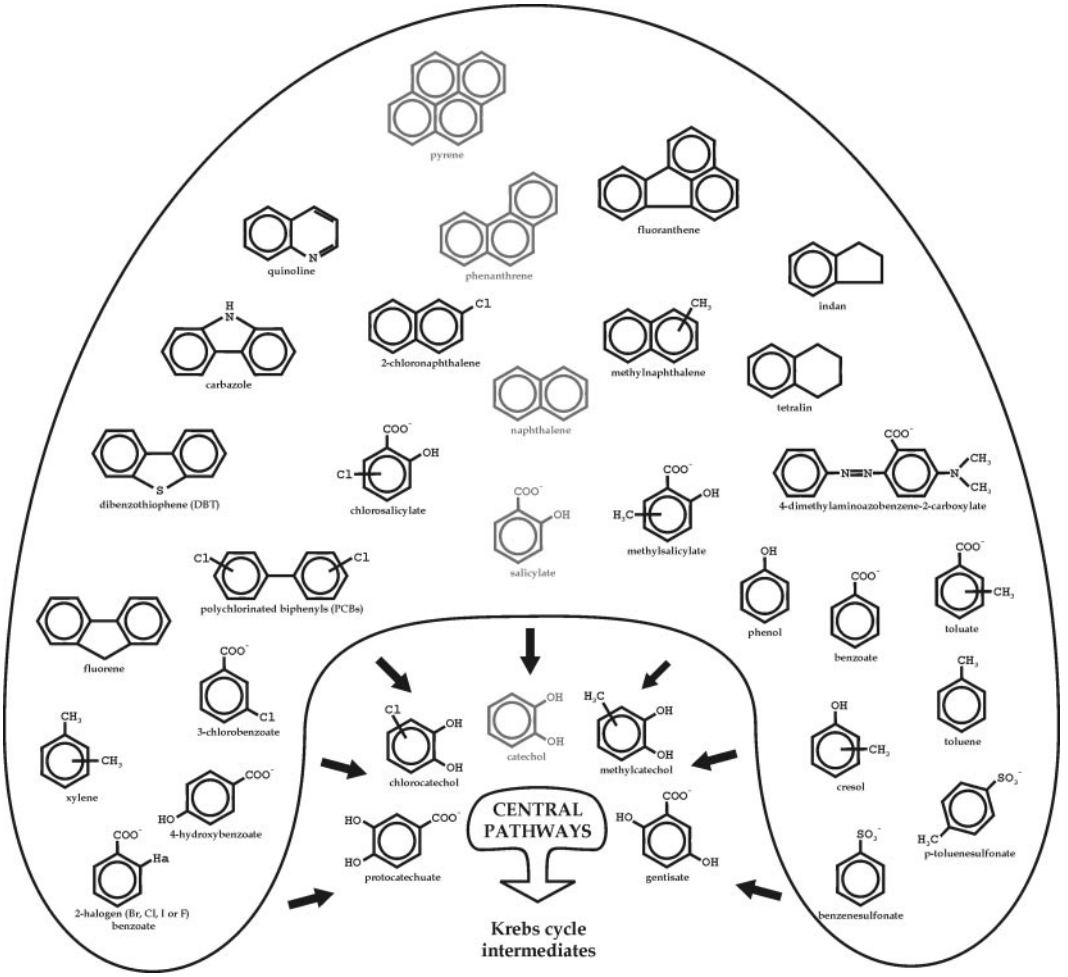

FIG. 4. Aerobic catabolism of aromatic compounds in *P. stutzeri*. The aerobic catabolism of these compounds involves a wide variety of peripheral degradation pathways that channel substrates into a small number of common intermediates (catechol, methylcatechol, chlorocatechol, protocatechuate, and gentisate). The intermediates are further processed by a few central pathways to tricarboxylic acid intermediates. Degradation of PAHs proceeds through reductions in the number of aromatic rings (i.e., pyrene [four rings] is channeled to phenanthrene [three rings]-, naphthalene [two rings]-, and salicylate [one ring]-like structures. These compounds are indicated in gray).

.atsdr.cdc.gov/cercla/). This list was drawn up by the EPA and the Agency for Toxic Substances and Disease Registry. The ability of *Pseudomonas* species to aerobically degrade benzene and its relatives is well known (164, 175, 237, 264, 364, 398). As shown in Fig. 4, the aerobic catabolism of these compounds involves a wide variety of peripheral degradation pathways. These pathways channel substrates into a small number of common intermediates (catechol, methylcatechol, chlorocatechol, protocatechuate, and gentisate). The intermediates are further processed by a few central pathways to tricarboxylic acid intermediates. The literature indicates that *P. stutzeri* strains are able to metabolize benzoate (2, 113, 311, 350); mono- and di -halogen Br, Cl, I, or F benzoates (188); 4-hydroxybenzoate (2, 311); benzenesulfonate and 4-methyl-benzenesulfonate (15); carbazole (151, 245); cresol (13); dibenzothiophene (150); fluoranthene (178); fluorene (349); indan (349); naphthalene (113, 114, 218, 296, 311, 349) and its methyl (113, 114, 349) and chloro (114) derivatives; PCBs (90, 107); phenanthrene (218, 348); phenol (2) and dimethylphenol (13); pyrene (177); quinoline (320); salicylate (113, 114, 311) and its methyl (113, 114) and chloro (114) derivatives; tetralin (311); toluate (113, 296, 311); toluene (13); and xylene (97, 113, 296). However, several environments, such as subsurface organic-impacted sediments, are commonly anaerobic. Thus, biodegradation in these anaerobic environments must occur in the absence of oxygen. Information on aerobic degradation of aromatic compounds is much more abundant than information on anaerobic degradation (reviews of anaerobic degradation of aromatic hydrocarbons can be found in references 142, 339, 396, and 219). However, several strains of *P. stutzeri* have been isolated under nitrate-reducing conditions as degraders of dibenzothiophene, pyrene, phenanthrene, naphthalene, benzoate, fluorobenzoate, and/or salicylate (150, 218, 286, 378).

The most-studied "*P. stutzeri*" strain is the toluene/xylene-degradative strain OX1 (9–11, 13, 17, 32, 33, 40, 58, 97, 197, 302, 303, 306, 312, 334, 353, 372–377). However, as mentioned above, this strain does not belong to the *P. stutzeri* species (see

"Definition of the species and differentiation from other *Pseudomonas* species," above). Two distinct *P. stutzeri* strains have been well studied due to their biological and biotechnological interest: strain P16 and strain AN10.

P. stutzeri strain P16 is a PAH-degrading bacterium. It was isolated from a phenanthrene enrichment culture of a creosote-contaminated soil (348). Strain P16 is able to grow, via salicylate, using phenanthrene (three rings), fluorene (two rings), naphthalene, and methylnaphthalenes (two rings) as the only carbon and energy sources (348, 349). It is also able to transform pyrene (four rings) to nonmineral products (177). Interestingly, the phenanthrene bacterial growth rate increased in the presence of Tergitol NP10, an anionic surfactant. The combination of strain P16, phenanthrene, and Tergitol has been proposed as a model for understanding the physical-chemical effects of surfactants on nonaqueous hydrocarbon bioavailability (130).

P. stutzeri strain AN10 is a naphthalene-degrading bacterium isolated from polluted marine sediments in the western Mediterranean Sea (113). Strain AN10 is able to dissimilate naphthalene, 2-methylnaphthalene, and salicylate as sole carbon and energy sources (295). In contrast to the usual plasmid location of the naphthalene-catabolic pathway (397, 398), its dissimilatory genes are chromosomally encoded (296). Its entire naphthalene degradation pathway has been cloned and sequenced. It is organized into four operons: one coding for the enzymes involved in the conversion of naphthalene to salicylate (*nahA*$_a$*A*$_b$*A*$_c$*A*$_d$*BFCED*) (42), two coding for the conversion of salicylate to pyruvate and acetyl-coenzyme A through the *meta* cleavage pathway enzymes (*nahW* and *nahGTHINLOMKJ*) (43, 44), and the last containing the regulatory gene *nahR* (44). Interestingly, two of these genes, *nahG* and *nahW*, encode two independent, inducible salicylate 1-hydroxylases (43). The gene *nahW* is unique to *P. stutzeri*. The two salicylate 1-hdyroxylases (NahG and NahW) from *P. stutzeri* AN10 were expressed upon incubation with salicylate. They are involved in naphthalene and salicylate metabolism (43). Both enzymes exhibited broad substrate specificities and metabolized salicylate, methylsalicylates, and chlorosalicylates. However, the relative rates at which the substituted analogs were transformed differed considerably. NahW was better at converting 3-chlorosalicylate, whereas NahG was more efficient at metabolizing methylsalicylates (43).

Biocides. A biocide is a chemical agent that, under carefully controlled conditions, can kill organisms on objects and materials. Biocides are used extensively (in agricultural, clinical, and industrial fields, etc.). The amount of biocides released from human activities into the environment is extremely large (e.g., the release of cyanide from industry has been estimated to be above 14 million kg per year [103]). Biocides persist in nature and remain as a potential source of pollution. Bacterial populations could be useful in detoxifying these agents. Biocides that are degraded and/or resisted by *P. stutzeri* strains are tributyltin (167, 168, 299), an organostannic compound used industrially as a stabilizer in plastics and wood preservatives and as an antifouling agent in boat paints; nonoxidizing industrial water treatment bactericides (52–54, 194), used in industrial water cooling systems to avoid microbially induced corrosion of metal surfaces; β-cyfluthrin (304), a pesticide used in agriculture to control lepidopteran pests affecting solanaceous crops; and cyanide and thiocyanates (129, 174, 389–391), used in petrochemical refining, the synthesis of organic chemicals and plastics, electroplating, aluminum works, and metal mining.

Of all *P. stutzeri* strains involved in biocide resistance and/or degradation, two are of biological and biotechnological interest: strain 5MP1 and strain AK61.

P. stutzeri 5MP1 is a tributyltin-resistant strain (MIC $>$ 1,000 mg $\cdot$ liter^{-1}) isolated from the sediment of Arcachon Harbor (France) (167). Tributyltin resistance was found to be associated with the presence of the operon *tbtABM*. It is a member of the resistance-nodulation-cell division efflux pump family (168). Interestingly, TbtABM conferred a multidrug resistance phenotype to strain 5MP1, including resistance to *n*-hexane, nalidixic acid, chloramphenicol, and sulfamethoxazole (168).

Many bacterial strains are capable of reducing the incidence of plant diseases caused by soilborne organisms such as bacteria, fungi, and nematodes. Such bacteria therefore act as biocontrol agents. Production of cyanide (HCN) by means of hydrogen cyanide synthetase has been studied intensively as one of the antibiosis mechanisms in the rhizosphere. Cyanide is highly toxic to living organisms because it inactivates the respiration system by tightly binding to cytochrome *c* oxidase (335). Some HCN-producing *Pseudomonas* species are plant beneficial, and others are plant deleterious. Such *Pseudomonas* species are common in soils. Thus, it is not surprising that bacteria have evolved with the capacity to degrade or detoxify HCN. *P. stutzeri* strain AK61 was isolated from wastewater at a metal-plating plant and was classified phenotypically and chemotaxonomically as a member of *P. stutzeri* (390). The aim of this study was to develop a biological treatment for cyanide. Such a treatment is needed, as cyanide is toxic and used in large amounts in the metal-plating, pharmaceutical, and agricultural-chemical industries. The biological treatment of cyanide may be cheaper and more environmentally acceptable than chemical methods such as alkaline chlorination, ozonization, and wet-air oxidation (100, 275). Whole cells of strain AK61 degraded cyanide rapidly in a 1 mM solution containing no organic substances. Induction of the cyanide-degrading activity was not dependent on the presence of cyanide. The cyanide-degrading enzyme was purified and characterized, and its encoding gene and potential active site were identified (389–391). Results indicate that the only enzyme responsible for the hydrolysis of cyanide to ammonia and formate was the cyanide-degrading nitrilase (cyanidase). More recently, the quaternary structure of the cyanide-degrading nitrilase from strain AK61 was determined. It is considered to be the model enzyme of the nitrilase superfamily (318). Enzymes from the nitrilase superfamily hydrolyze and condense a variety of nonpeptide carbon-nitrogen bonds (49, 247). As a result, there is considerable interest in these enzymes as industrial catalysts. Therefore, uses include the production of nicotinic acid, *R*-(−)-mandelic acid, and *S*-(+)-ibuprofen and the detoxification of cyanide waste (78).

P. stutzeri is also involved in cometabolic degradative processes. A *P. stutzeri* strain was isolated from chemostat enrichment on bacteria that degrade the organophosphate insecticide parathion. This strain was able to cleave the substrate in *p*-nitrophenol and diethylthiophosphate but could not use either of the resulting molecules (234). Another *P. aeruginosa* strain in the consortium can mineralize *p*-nitrophenol

but cannot attack intact parathion. The two-component enrichment degrades parathion synergistically with high efficiency. *P. stutzeri* apparently utilizes the products excreted by *P. aeruginosa*.

Proteolytic activity: applications for biorestoration. *P. stutzeri* is not considered to be proteolytic, as discussed in "Phenotypic identification," above. Only 1% of the strains give a positive reaction in the gelatinase test. However, *P. stutzeri* strain A29 was selected from a group of other *Pseudomonas* strains, as it exhibited good proteolytic activity in culture supernatant (273).

The aim of the study was to select the best proteolytic bacterium for hydrolyzing the insoluble animal glue on a fresco called *Conversione di S. Efisio e Battaglia*, painted by Spinello Aretino (1391 to 1392). The most abundant components in animal glue are collagen and casein. The fresco was removed from its wall by a technique that involved the use of animal glue as a consolidating agent and treatment with formaldehyde as an antimicrobial agent. After the fresco was removed, the front of it was treated with proteolytic enzymes to restore the painting to view. The usual proteolytic treatments did not work. However, spraying of the fresco with a high density of viable *P. stutzeri* cells resulted in a satisfactory biorestoration process in 10 to 12 h. The most abundant proteolytic enzyme was 120 kDa in size and showed collagenase and caseinolytic activities. This enzyme has been studied in detail (7). The current working hypothesis is that different proteases with unique activities may act cooperatively.

NATURAL TRANSFORMATION

Genome analysis and molecular microbial ecology studies have shown that horizontal gene transfer is a relevant force in bacteria for continuous adaptation to environmental changes. Three broad mechanisms mediate the efficient movement of DNA between cells: transduction, conjugation, and natural transformation. Natural transformation involves bacterial uptake of naked DNA from the surrounding environment and its integration into the genome. Natural transformation has been observed in the bacterial species of very different phylogenetic and trophic groups. Natural transformation is perhaps the most versatile mechanism of horizontal gene transfer (206). *Pseudomonas stutzeri* can be considered a naturally transformable bacterium, as one-third of its members are naturally transformable (60, 207, 326). Its transformation capability has been extensively studied during the last two decades. Competence is not constitutive in most naturally transformable bacteria; it depends on physiological state. *P. stutzeri* competence occurs in broth-grown cultures during the transition from the log phase to the stationary phase (60, 205). *P. stutzeri* competence is also developed in media prepared from aqueous extracts of various soils (204, 205). It is further stimulated under carbon-, nitrogen-, and phosphorous-limited conditions (204, 205), such as those frequently encountered by bacteria in soil. It has been demonstrated that *P. stutzeri* can be transformed by mineral-associated DNA in laboratory-designed glass columns (203), DNA bound in autoclaved marine sediment (342), and DNA adsorbed in sterilized soil (250). *P. stutzeri* can also access and take up DNA bound to soil particles in the presence of indigenous DNases, in competition with native microorganisms (323).

P. stutzeri can be transformed by chromosomal and plasmid DNA. However, initial studies considered transformation only in the presence of homologous DNA, speculating that recognition sequences were necessary for DNA uptake (60, 61). Later studies reported natural transformation by *P. stutzeri* with different broad-host-range plasmids formed only by heterologous DNA (207, 326). Thus, it can be concluded that competent cells of *P. stutzeri* take up foreign DNA as well as DNA from their own species. However, the frequency of foreign DNA acquisition events was only 0.0003% of the value observed for fully homologous DNA transformation (221). The presence of a short (311-bp) homologous sequence on one side of the foreign DNA increased this frequency by 200-fold. However, gene integration occurred mostly in the nonhomologous region, with the help of an illegitimate recombination event involving 3- to 6-bp G+C-rich microhomologies (221). In addition, a *recA* mutation decreased transformation with one-sided homologous DNA by at least 100-fold (221). These results suggest that genomic acquisition of foreign DNA by *recA*-dependent illegitimate recombination occurs in *P. stutzeri*.

Transformability is widespread among environmental *P. stutzeri* strains. However, it has been shown that nontransformability and different levels of transformability are often associated with distinct genomic groups (326). This suggests that transformation capability may be associated with speciation in the highly diverse species *P. stutzeri*. In this respect, it has been shown that the presence of DNA restriction-modification systems and mismatch repair mechanisms in *P. stutzeri* act as barriers to the uptake of foreign DNA. These mechanisms may therefore contribute to sexual isolation and further speciation (31, 222).

Natural transformation capability requires the presence of a considerable number of gene products. Although much information has been obtained for *Bacillus subtilis* and *Neisseria gonorrhoeae*—see a review by Chen and Dubnau (67)—the transformation machinery of *P. stutzeri* has been studied only recently (125–128, 220). It has been demonstrated that *P. stutzeri* naturally transforms both duplex and single-stranded DNA using the same machinery. The levels of duplex DNA transformation are 20- to 60-fold higher than the levels of single-stranded DNA transformation (220).

It has been reported that type IV pili are essential to genetic transformation in *P. stutzeri* (125). In this study it was shown that insertional inactivation of two genes, *pilAI* and *pilC*, abolished pilus formation. In addition, mutants of both genes were not able to transform DNA. The *pilAI* gene showed high similarity to pilin genes of other species. Its product, PilAI, was defined as the structural protein of the *P. stutzeri* type IV pili. PilAI was involved in the first step of transformation: the competence-specific binding of duplex DNA, its transport into the periplasm, and its transformation in a DNase-resistant state (125). The *pilC* gene of *P. stutzeri* is transcribed with two other *pil* genes, *pilB* and *pilD*. Its product, PilC, was shown to be essential for DNA transformation. It seems to be a hydrophobic protein involved in the transport of processed PilAI protein (125). The *pilB* and *pilD* gene products, PilB and PilD, resemble accessory proteins in type IV pilus biogenesis. They are probably located in the cytoplasm and in the inner membrane, respectively (125). Interestingly, a new gene, *pilAII*, was identified downstream from the *pilAI* gene. Its product, PilAII,

is 55% identical in amino acid sequence to that of PilAI (127). Although both genes were cotranscribed, the expression of *pilAII* was only 10% of that observed for *pilAI* (127). Secondary pilin-coding genes have been found in other well-studied transformable bacteria, such as *Neisseria gonorrhoeae*, *Acinetobacter* sp. strain BD4, *Bacillus subtilis*, *Streptococcus pneumoniae*, and *S. gordonii*. Their inactivation results in a loss of transformation capability (56, 71, 210, 262, 403). Surprisingly, the genetic inactivation of *P. stutzeri pilAII* produced a hypertransformation phenotype (127). It has been suggested that the role of PilAII is to interfere with DNA transport within the cell following DNA uptake. PilAII therefore acts as a factor that is antagonistic to genetic transformation. Its controlled expression defines the level of transformability shown by naturally competent *P. stutzeri* cells (127).

The second step of transformation consists of the translocation of DNA from the periplasm to the cytoplasm. In *P. stutzeri*, this step is totally dependent on the *comA* gene product (126). ComA is a polytopic integral membrane protein that is thought to form the pore through which single-stranded DNA reaches the cytoplasm (126). The nuclease involved in the transformation of duplex DNA into a single-stranded molecule remains unknown (67). No ATP-binding site has been found in the ComA amino acid sequence. This suggests that ComA is not the driving force behind DNA translocation. Instead, ComA may act in a protein complex with an energy-supplying enzyme (126). Inactivation in *P. stutzeri* of the *exbB* gene led to a reduction in its natural transformation rate (126). The product of *exbB* has been described as a member of the TonB-ExbB-ExbD complex (126). In *E. coli*, this complex is thought to mediate energy transfer of the electrochemical potential from the cytoplasm to the periplasm (193). Thus, it has been suggested that ExbB interacts with ComA in *P. stutzeri* to supply the energy needed for DNA translocation (126).

Finally, two other cotranscribed genes, *pilT* and *pilU*, have been identified and shown to be required for full transformability of *P. stutzeri* (128). In fact, *pilT* inactivation produces a transformation-deficient strain that is unable to take up DNA. A *pilU* mutant was only 10% naturally transformable compared with the wild-type strain (128). Both gene products, PilT and PilU, are homologous to components of a specialized protein assembly system—competence traffic NTPases—that is widely found in bacteria. This system is responsible for depolymerizing the pilus into pilin monomers. Consequently, it is also responsible for pilus retraction (387). Thus, it has been suggested that pilus retraction pulls DNA into the periplasm from the bacterial surface. Subsequently, DNA is somehow moved to the ComA complex, where one strand is degraded. The resulting single-stranded DNA is finally translocated into the cytoplasm (128).

PATHOGENICITY AND ANTIBIOTIC RESISTANCE

For a 15-year period after 1956, several reports described the isolation of *P. stutzeri* from clinical and pathological materials. However, there was no clear association of this species with an infectious process (117, 118, 182, 191, 260, 340, 394). In fact, 15 of the 17 strains studied in 1966 by Stanier et al. (340) were of clinical origin. In 1973, the first well-documented case of *P. stutzeri* infection appeared in the literature. It involved a nonunion fracture of a tibia (119). Since then, a few cases of *P. stutzeri* infection have been reported in association with bacteremia/septicemia (124, 180, 266, 267, 379); bone infection, i.e., fracture infection, joint infection, osteomyelitis, and arthritis (119, 211, 279, 298, 361); endocarditis (290); eye infection, i.e., endophthalmitis and panophthalmitis (165, 195); meningitis (287, 354); pneumonia and/or empyema (59, 62, 187, 244, 266, 317, 407); skin infection, i.e., ecthyma gangrenosum (269); urinary tract infection (352); and ventriculitis (381). Only two of the above cases resulted in death (62, 180). This reflects *P. stutzeri*'s relatively low degree of virulence. In fact, it is doubtful whether death was due to *P. stutzeri* infection in these two cases, as both patients had severe malfunctions caused by underlying conditions: chronic renal failure (180) and chronic liver disease (62). Interestingly, almost all patients with the aforementioned *P. stutzeri* infections had one or more of the following predisposing risk factors: (i) underlying illness, (ii) previous surgery (implying probable nosocomial acquisition), (iii) previous trauma or skin infection, and (iv) immunocompromise. Only two cases lacked any of these known risk factors: a man with vertebral osteomyelitis (279) and a 4-year-old boy with pneumonia and empyema (187).

Studies to determine the distribution rates of *P. stutzeri* in hospitals have also been carried out. Two different studies were undertaken with all of the bacterial isolates obtained in university hospitals during a defined period from samples of wound pus, blood, urine, tracheal aspirates, and sputum. Both studies concluded that 1 to 2% of all the *Pseudomonas* spp. isolated were *P. stutzeri* (104, 238). Similar isolation rates (1.8%) were obtained in a study of *Pseudomonas* sp. infections in patients with human immunodeficiency virus disease (213). Interestingly, the highest rate of *P. stutzeri* isolation was reported by Tan et al. (352), who showed that 3% of all urine-isolated bacteria were *P. stutzeri*. Thus, it can be concluded that *P. stutzeri* is also ubiquitous in hospital environments and that this species could be considered an opportunistic but rare pathogen.

Sensitivity tests for several antibiotics were performed in nearly all of the epidemiological and case reports mentioned above. There is a summary of these studies in Table 2. Nearly all studies involving several antibiotics and bacterial species showed that *P. stutzeri* was sensitive to many more antibiotics than *P. aeruginosa*, its most closely related species and a well-known human pathogen (238, 352, 356). Its higher sensitivity was explained by its reduced occurrence in clinical environments and, consequently, its lower exposure to antibiotics. In spite of these results, when bacterial isolates were obtained from immunosuppressed patients (i.e., patients with human immunodeficiency virus disease) no significant differences in antibiotic susceptibility between *P. aeruginosa* and other *Pseudomonas* spp., including *P. stutzeri*, were detected (213). Immunosuppressed patients are normally hospitalized for long periods. They are generally in contact with more types of antibiotics at higher doses. This extensive use of antibiotics could be responsible for the higher rate of isolation of antibiotic-resistant *P. stutzeri* strains. Interestingly, with the exception of fluoroquinolones, resistant *P. stutzeri* strains have been isolated for almost all antibiotic families (Table 2). This suggests that *P. stutzeri* has a wide range of antibiotic resistance mechanisms. At least two such antibiotic resistance mechanisms in

TABLE 2. Antibiotic sensitivities of *P. stutzeri* strains

Family	Antibiotic	Test results by yr (no. of isolates analyzed)[a]												
		1970 (22)	1972 (32)	1974 (17)	1977 (41)	1983 (34)	1987 (2)	1994 (16)	1997 (40)	1998 (1)	1999 (6)	2000 (46)	2004 (1)	All (258)
Narrow-spectrum fluoroquinolones	Nalidixic acid	—	S	—	S	—	—	—	—	—	S	—	—	S
Extended-spectrum fluoroquinolones	Ciprofloxacin	—	—	—	—	—	—	S	S	—	—	S	S	S
	Norfloxacin	—	—	—	—	—	S	—	S	—	—	—	—	S
	Ofloxacin	—	—	—	—	—	—	—	S	S	—	S	—	S
Broad-spectrum fluoroquinolones	BMS-284756	—	—	—	—	—	—	—	—	—	—	S	—	S
	Clinafloxacin	—	—	—	—	—	—	—	S	—	—	—	—	S
	Gatifloxacin	—	—	—	—	—	—	—	—	—	—	S	—	S
	Levofloxacin	—	—	—	—	—	—	—	S	—	—	S	—	S
	Moxifloxacin	—	—	—	—	—	—	—	—	—	—	S	—	S
	Sparfloxacin	—	—	—	—	—	—	—	S	—	—	—	—	S
	Trovafloxacin	—	—	—	—	—	—	—	S	—	—	S	—	S
Aminoglycosides	Amikacin	—	—	—	—	—	S	—	S	—	—	—	S	S
	Aminosidine	—	—	—	S	—	—	—	—	—	—	—	—	S
	Gentamicin	S	S	R	S	R	S	—	S	S	S	—	—	R
	Kanamycin	S	S	R	S	—	—	—	—	—	S	—	—	R
	Neomycin	—	S	—	S	—	—	—	—	—	S	—	—	S
	Netilmicin	—	—	—	—	—	S	—	—	—	—	—	S	S
	Streptomycin	—	S	—	R	—	—	—	—	—	R	—	—	R
	Tobramycin	—	—	S	—	—	S	—	S	—	—	—	—	S
β-Lactam, narrow-spectrum cephalosporins	Cefazolin	—	—	—	—	R	R	—	R	—	—	—	—	R
	Cephaloridine	—	—	R	—	—	—	—	—	—	—	—	—	R
	Cephalothin	—	R	—	R	—	—	—	R	—	—	—	—	R
β-Lactam, extended-spectrum cephalosporins	Cefamandole	—	—	—	—	—	—	—	—	—	R	—	—	R
	Cefoxitin	—	—	—	—	—	—	—	R	—	—	—	—	R
	Cefuroxime	—	—	—	—	—	R	—	R	—	R	—	—	R
	Cephacetrile	—	—	R	—	—	—	—	—	—	—	—	—	R
β-Lactam, broad-spectrum cephalosporins	Cefotaxime	—	—	—	—	—	S	—	S	—	—	—	S	S
	Ceftazidime	—	—	—	—	—	S	S	R	S	—	—	—	R
	Moxalactam	—	—	—	—	—	S	—	—	—	—	—	—	S
	Cefepime	—	—	—	—	—	—	—	—	—	—	—	S	S
β-Lactam carbapenems	Imipenem	—	—	—	—	—	—	—	S	—	—	—	—	S
	Meropenem	—	—	—	—	—	—	—	—	—	—	—	S	S
β-Lactam, extended-spectrum penicillins	Ampicillin	S	S	R	S	R	S	—	R	—	R	—	—	R
	Carbenicillin	S	—	S	S	S	—	—	—	—	—	—	—	S
	Mezlocillin	—	—	—	—	—	S	—	—	—	—	—	—	S
	Piperacillin	—	—	—	—	—	—	—	S	S	—	—	S	S
	Ticarcillin	—	—	—	—	—	S	—	—	—	—	—	—	S
Extended-spectrum penicillins/ β-lactamase inhibitors	Amoxicillin/ Clavulanic acid	—	—	—	—	—	—	—	R	—	—	—	—	R
	Ticarcillin/ Clavulanic acid	—	—	—	—	—	—	—	S	—	—	—	—	S
β-Lactam monobactams	Aztreonam	—	—	—	—	—	S	—	R	—	—	—	—	R
	BMS-180680	—	—	—	—	—	—	—	R	—	—	—	—	R
β-Lactam natural penicillins	Penicillin G	—	R	—	R	—	R	S	S	—	—	—	—	R
β-Lactam semisynthetic penicillins	Azlocillin	—	—	—	—	—	S	—	—	—	—	—	—	S
	Cloxacillin	—	—	—	—	—	—	—	—	—	R	—	—	R
	Methicillin	—	—	—	S	—	—	—	—	—	R	—	—	R
	Oxacillin	—	—	—	—	—	—	—	S	—	—	—	—	S

Continued on following page

TABLE 2—*Continued*

Family	Antibiotic	Test results by yr (no. of isolates analyzed)[a]												
		1970 (22)	1972 (32)	1974 (17)	1977 (41)	1983 (34)	1987 (2)	1994 (16)	1997 (40)	1998 (1)	1999 (6)	2000 (46)	2004 (1)	All (258)
Coumarins	Novobiocin	—	R	—	R	—	—	—	—	—	R	—	—	R
Glycopeptides	Vancomycin	—	—	—	R	—	—	—	—	—	R	—	—	R
Lincosamides	Mandelamine	—	—	—	S	—	—	—	—	—	—	—	—	S
Macrolides	Erythromycin	S	S	—	S	—	—	R	R	—	R	—	—	R
	Lincomycin	—	R	—	R	—	—	—	—	—	—	—	—	R
Polymyxins	Colistin	S	—	—	R	—	—	—	—	—	—	—	—	R
Sulfonamides	Bactrim/Septra	—	—	—	S	—	—	—	—	—	—	—	—	S
	Co-trimoxazole	—	—	—	—	—	—	—	R	—	—	—	—	R
	Sulphatriad	—	—	—	S	—	—	—	—	—	—	—	—	S
	Trimethoprim-sulfamethoxazole	—	—	—	—	—	S	S	S	—	—	—	—	S
Tetracyclines	Doxycycline	—	—	—	—	—	S	—	—	—	—	—	—	S
	Minocycline	—	—	—	—	—	S	—	—	—	—	—	—	S
	Tetracycline	S	S	R	S	R	—	—	S	S	S	—	—	R
Miscellaneous antibiotics	Chloramphenicol	S	S	S	S	R	R	R	R	—	—	—	—	R
	Clindamycin	—	—	—	—	—	—	—	S	—	—	—	—	S
	Fusidic acid	—	—	—	—	—	—	—	—	—	R	—	—	R
	Nitrofurantoin	R	R	—	R	—	—	—	—	—	—	—	—	R
	Polymyxin B	S	S	S	R	S	S	—	—	—	S	—	—	R
	Rifampin	—	—	—	—	—	—	—	—	—	R	—	—	R

[a] S, all strains analyzed were sensitive; R, one or more strains were resistant; —, not tested. References to studies from each year are as follows: 1970 (260), 1972 (118), 1974 (211, 301), 1977 (82, 352), 1983 (180, 317), 1987 (266, 290), 1994 (238, 257), 1997 (59, 110, 256, 383), 1998 (165), 1999 (356, 357), 2000 (111, 112), 2004 (187).

P. stutzeri have been described: (i) alterations in outer membrane proteins and lipopolysaccharide profiles (357–359) and (ii) the presence of β-lactamases that hydrolyze natural and semisynthetic penicillins, broad-spectrum "β-lactamase-stable" cephalosporins, and monobactams with similar rates (108).

HABITATS AND ECOLOGICAL RELEVANCE

The remarkable physiological and biochemical diversity and flexibility of *P. stutzeri* is shown by its capacity to grow organotrophically through mineralizing or degrading a wide range of organic substrates; its ability to grow anaerobically, using different terminal electron acceptors in a strictly oxidative metabolism; its oxidation of inorganic substrates, as a chemolithotrophic way to gain accessory energy; its resistance to heavy metals; and the variety of nitrogen sources it can use. We have discussed how *P. stutzeri* participates in key processes of element cycling, including C, N, S, and P. In addition, a wide range of temperatures support *P. stutzeri* growth. This is an important physiological characteristic when the habitats that can be colonized by this species are considered. Phenotypic heterogeneity may be explained by *P. stutzeri*'s huge range of habitats and growth conditions, including the human body. Spiers et al. classified ecological opportunity and competition as the main ecological causes of diversity (338). They emphasized that the underlying cause of diversity is genetic and that diversification occurs through mutation and recombination. The natural competence demonstrated by many *P. stutzeri* strains can help to increase genetic diversity. It provides new genetic combinations for colonizing new habitats or for occupying new ecological niches, even when the population is essentially clonal. It has insertion sequences, and mosaic gene structures have also been reported. There is considerable variation in the length of its genome (121). All of these factors suggest that different events may contribute to overall species diversity. The presence of *P. stutzeri* is almost universal. It has been detected through specific DNA sequences extracted directly from environmental samples (*nirS*, *nosZ*, *nifH*, 16S rRNA). It has also been isolated intentionally or accidentally from many habitats. Some of these, including extreme habitats, are considered below.

Soil, Rhizosphere, and Groundwater

The composition of the bacterial rhizosphere population, and in particular that of the diazotrophic bacteria, is of major interest. New isolation media and enrichment conditions have been developed with low oxygen tensions simulating rhizosphere conditions. This has led to the conclusion that the genus *Pseudomonas* is dominant or predominant in association with wheat, barley, and wetland rice (66, 93, 208). The role of diazotrophic *P. stutzeri* strains in soils might be more relevant than previously considered. A recent study involving PCR and denaturing gradient gel electrophoresis analysis of the N_2-fixing bacterial diversity in soil revealed a high percentage of *nifH* genes identical to those of *P. stutzeri* (93). Molecular

analysis of diazotroph diversity in the rhizosphere of smooth cordgrass (*Spartina alterniflora*) suggests that *P. stutzeri*-related strains are present in the *Spartina* rhizosphere. Recently, analysis of bacterial populations in the rhizosphere of cordgrass, based on PCR amplification of *nifH* sequences and separation of the amplicons by denaturing gel electrophoresis, revealed *nifH* sequences highly similar to those of strains A1501 (a derivative of strain A15) and CMT.9.A (208, 209). The activity of an aromatic amino acid aminotransferase and the production of indole-3-acetic acid in *P. stutzeri* A15 have also been reported. This may be involved in the production of growth-regulating substances in plants in addition to their nitrogen-fixing ability (261).

As mentioned above, many *P. stutzeri* strains have been isolated from contaminated soil sites, where degradative and contaminant-resistant strains have to develop relevant ecological activities. Some strains, such as KC, and several methyl-naphthalene-degradative strains have been isolated in our laboratory from groundwaters contaminated with aircraft fuel (JetA1). The efficacy of strain KC in detoxifying groundwaters has been shown through bioaugmentation.

Marine Water and Sediment and Salt Marshes

Most strains isolated from marine environments and initially classified in the genus *Pseudomonas* have been transferred to other genera after an analysis of their phylogenies. These transfers include *P. doudoroffii* to *Oceanimonas doudoroffii*, *P. nautica* to *Marinobacter hydrocarbonoclasticus*, *P. stanieri* to *Marinobacterium stanieri*, *P. elongata* to *Microbulbifer hydrolyticus*, and *P. marina* to *Cobetia marina*. Not many species within the genus *Pseudomonas* sensu stricto have been detected in marine waters. For a strain to be considered of marine origin, it must have the physiological characteristic of requiring, or at least tolerating, NaCl. *P. stutzeri* (including strain ZoBell, formerly *P. perfectomarina*), *P. balearica*, and *P. xanthomarina* (isolated from ascidian specimens in the Sea of Japan [289]) seem to be true marine *Pseudomonas* species. In addition, *P. alcaliphila* and *P. aeruginosa* (181) have been isolated from marine waters. Further research is required to define whether the latter pseudomonads might be considered marine bacteria or allochthonous to the ecosystem.

Marine strains of *P. stutzeri* are located in the water column and in sediment. The most relevant strains studied in detail are ZoBell (isolated from the water column in the Pacific ocean and studied as a model denitrifier in marine environments), AN10 (isolated from polluted Mediterranean marine sediment and studied as a naphthalene degrader), NF13 (isolated from a sample taken at 2,500- to 2,600-m depth in the Galapagos rift from near a hydrothermal vent and studied as a strain that oxidizes sulfur chemolithotrophically), and strains MT-1 and HTA208 (isolated from deep-sea samples taken at the Mariana Trench at 10,897-m depth). The main ecological role of these strains seems to be denitrification, besides their specific physiological properties.

The study by Sikorski et al. (325) is the only one in which a large number of *P. stutzeri* strains have been isolated from the same sample, in this case marine sediment from the shore of the North Sea. This enabled a genetic study of the populations present in a single habitat to be undertaken.

The ability of *P. stutzeri* to oxidize thiosulfate to tetrathionate both aerobically and anaerobically was not known before the work of Sorokin et al. (337). Several strains were isolated from the Black Sea at more than 100 m in depth. It was suggested that this widespread bacterium could be important in the turnover of thiosulfate in marine environments and that it may compete with thiosulfate disproportionation and reduction by thiosulfate-reducing bacteria.

Spartina marshes support high rates of macrophyte primary production and microbially mediated nutrient cycling. The possible ecological role of *P. stutzeri* in such marshes seems to be its contribution to global carbon and nitrogen budgets. Primary production and decomposition in *Spartina* marshes are nitrogen limited (208). In these systems, diazotrophy is a key source of new nitrogen, and denitrification completes the nitrogen cycle. *P. stutzeri* participates in both processes. Direct molecular analysis of diazotrophic diversity in the rhizosphere of *Spartina alterniflora* demonstrates that gene sequences of *nifH* are highly similar to those of *P. stutzeri*. In addition, they are located in the same phylogenetic branch as many other sequences of *nif* genes obtained from marine microorganisms.

Wastewater Treatment Plants

To screen bacteria with unusual metabolic properties, such as the degradation of anthropogenic compounds for bioremediation purposes, it is common to examine samples taken from wastewater treatment plants or to design bioreactors imitating the conditions of a treatment plant. Naphthalene degraders, thiosulfate oxidizers, chlorobenzoate degraders, and cyanide oxidizers have been isolated in this way. It has been demonstrated that *P. stutzeri* is also distributed in wastewater. However, no attempt has been made to quantify *P. stutzeri* in such habitats or to determine its relevance.

CONCLUSIONS

P. stutzeri genomovars can be considered genomospecies, as defined by J. P. Euzéby. According to his recommendations, if a genomospecies has been identified it is possible to look for phenotypic traits that differentiate it from the other genomospecies. If the genomospecies can be identified phenotypically, it must receive a name and be converted into a new species. If no phenotypic characteristic can be used to identify the genomospecies easily, it is left without a name. We prefer to maintain the genomovar concept for the genomic groups in *P. stutzeri*, because all of them share the basic phenotypic traits of the species. If DNA-DNA similarity results, or a multigenic sequencing approach, are accepted as the only criteria for species delineation, then *P. stutzeri* should be split into 17 different species. However, in our opinion, this situation would not help to clarify the taxonomic position of a phylogenetic and phenotypically coherent group of strains, as is the case for members of *P. stutzeri*.

As demonstrated, genomovars are monophyletic biological and evolutionary units in which different ecotypes may be differentiated by their adaptation to new environmental conditions. *P. stutzeri* is widely distributed in natural environments and shows great metabolic versatility, which is consistent with a large effective population size. This species shows very low

recombination rates. When there is a large population size and no assortive recombination, bacterial clones diverge freely by accumulating neutral mutations. The occurrence in a particular population of adaptive mutations conferring selective advantages in specific ecological situations leads to the elimination of genetic diversity within the population. However, in the presence of very low recombination rates, such mutations do not prevent genetic divergence between populations. Thus, the exceptionally high genetic diversity of *P. stutzeri* may be the result of niche-specific selection that occurs during colonization and adaptation to a wide range of microenvironments. Horizontal gene transfer seems to be an efficient mechanism for introducing new phenotypes into the genomes of *P. stutzeri*, without affecting the housekeeping genes. Integrons may play an important role in the acquisition of these new properties.

In conclusion, *P. stutzeri* exhibits exceptionally high diversity within a clonal population structure. In such cases, the existence of a strong linkage disequilibrium can be explained by considering that *P. stutzeri* forms a metapopulation made up of multiple ecological populations. These populations occupy different ecological niches. Although recombination is possible within populations, it is rare or absent between different populations (216, 278, 410). More-extensive studies are required to assess the population structure of these ecological populations of *P. stutzeri*. However, the results reported to date are consistent with the conclusion that this bacterial species represents a good example of a phenotypically cosmopolitan ecological species sensu Istock (160), i.e., a species characterized by limited phenotypic variation, restricted local sets of genetic clones, and no or rare recombination. The clonal sets are genetically diverse, but phenotypic resemblance is sufficient to make phenetic classification and identification possible. *P. stutzeri* is the species with the highest genetic diversity described to date. MLEE and MLST data confirm the results obtained by other techniques that have shown that some clones of *P. stutzeri* are distinct enough to warrant taxonomic differentiation (23).

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